THE FERMENTATION OF ^{14}C-PLANT CELL WALLS IN THE RAT GASTROINTESTINAL TRACT

by

DOUGLAS FRASER GRAY

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To Mum and Dad

DECLARATION

I hereby declare that the work described in this thesis is my own and has not been accepted for any degree in a university or further education establishment. The work was carried out in the Department of Botany, Kings' Buildings, University of Edinburgh and the Gastrointestinal Laboratories, Western General Hospital in Edinburgh between October 1986-1989.

D. Fraser Gray (October 1989)

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My parents and family have been a constant source of support and understanding throughout my university career. Without their encouragement all this would not be possible.

ABBREVIATIONS

AHF	Acid Hydrolysed Fraction
Ara	Arabinose
BAW	Butan-1-ol/Acetic acid/water (12:3:5)
Bq	Becquerels
Ci	Curies
cm	Centimeter
cpm	Counts Per Minute
°C	Degrees Centigrade
DF	Dietary Fibre
DMSO	Dimethylsulphoxide
dpm	Disintegrations Per Minute
DSF	Driselase Soluble Fraction
ELF	Ester-Linked Fraction
EPW	Ethyl acetate/Pyridine/water (8:2:1)
Fuc	Fucose
g	Grams
Gal	Galactose
GalA	Galcturonic acid
G.I.	Gastrointestinal
Glc	Glucose
h	Hour
HCl	Hydrochloric acid
HEPES	N-2 Hydroxyethylpiperazine-N'-2-ethansulfonic acid
HVPE	High Voltage Paper Electrophoresis
l	Litre
Man	Mannose
mm	Millimeter
mmol	Millimole
NaOH	Sodium hydroxide
PAW	Phenol:Acetic acid:Water (2:1:1)
POPOP	2,5-Diphenyloxazole
PPO	1,4-Di-2-(5-phenyloxazoyl)
Rha	Rhamnose
RITA	Radioactivity-TLC-Analyser

rpm	Revolutions Per Minute
R_F	Relative Mobility
R_{Rhm}	Mobility Relative to Rhamnose
SDS	Sodium dodecasulphate
SCFA	Short Chain Fatty Acid
s. e. m.	Standard Error of the Mean
TFA	Trifluoroacetic acid
v/v	volume/volume
w/v	weight/volume
XG	Xyloglucan
XG2	Xylosyl-α(1→4)-glucose
XG9	Xyloglucan nonasaccharide
Xyl	Xylose

ABSTRACT

Dietary fibre (DF) has become an increasingly important part of a healthy diet as its fate in the gastrointestinal (G.I.) tract becomes apparent. The heterogeneous nature of DF components makes the understanding of the overall beneficial effects confusing. The methodology has been limited and in this thesis a new approach to investigating the fate of a radioactive dietary fibre marker in the G.I. tract of rats is described.

A U-^{14}C-plant cell wall preparation produced from spinach cell cultures was analysed by various chemical and enzymic techniques. The ^{14}C distribution in the plant cell wall was confined mainly to the major polysaccharide groups: pectins (48.4%), hemicelluloses (15.3%), cellulose (21.3%) and starch (2.6%). The pectins consisted of predominantly homogalacturonan and the major hemicellulose was xyloglucan. The analysis confirmed that the suspension cultured spinach cell walls were a good comparison with other plant cell walls. Therefore the U-^{14}C-plant cell wall preparation was used in animal studies as a marker for dietary fibre and its fate investigated.

The pectic fraction of the cell walls was degraded completely in the rat G.I. tract but the hemicellulose/cellulose fraction was still detected in the colon. It is postulated that the chemical and physical properties of different plant cell wall polysaccharides will dictate their fate in the caecum and colon with respect to degradation by the bacterial microflora.

The U-^{14}C-plant cell wall preparation was over 85% degraded by the bacterial microflora and the fermentation products were utilised by the host. The incorporation of ^{14}C into host tissues was high (22% of the ^{14}C dose) as was the production of $^{14}CO_2$ (26% of the ^{14}C dose) in high fibre fed animals. *In vitro* fermentation, using a caecal inoculum, confirmed the production of short chain fatty acids (SCFAs) with reduced $^{14}CO_2$ production (12% of ^{14}C dose). These studies show that both bacterial fermentation of ^{14}C-plant cell walls and host metabolism of ^{14}C-SCFAs result in the production of $^{14}CO_2$.

Comparative studies using animals maintained on low fibre diets showed that both the $^{14}CO_2$ production *in vivo* (16% of ^{14}C dose) and *in vitro* (7% of ^{14}C dose) were reduced. The level of SCFAs was also much lower in the low fibre fed animals suggesting that their caecal bacterial capacity to ferment polysaccharide was grossly reduced. By using the ratio of *in vitro* to *in vivo* production of $^{14}CO_2$ as an indicator of the host capacity to absorb and metabolise SCFAs it was clear that there was no difference between a high and low fibre diet, despite the differences between the activities of the bacterial populations.

To conclude, these U-^{14}C-labelled spinach cell walls are a good marker to investigate the fate of DF in the G.I. tract. There is a potential to use the methods described to extend the available information on the biological effects of dietary fibre in our diets.

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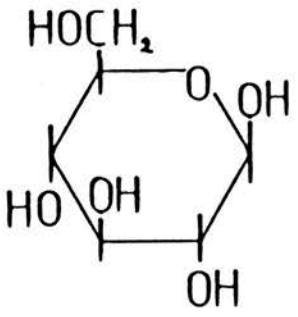
CHAPTER 1 : INTRODUCTION

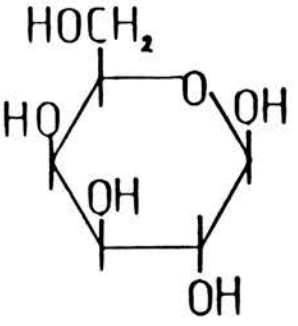
Composition of Dietary Fibre

The revised definition of dietary fibre (DF) proposed by Trowell *et al.* (1976), and now widely accepted, includes the "polysaccharides and lignin not digested by the endogenous secretions of the human gastrointestinal (G.I.) tract". The definition however is the focus for debate and this is discussed in chapter 5. Most of our DF intake comes from the cell walls of fruits, vegetables and cereals. The fruits and vegetables generally consumed contain mainly parenchymatous tissue with thin primary (growing) cell walls, whereas the cell walls of cereals and cereal based products are lignified. Although the roots (e.g. carrots), stems (e.g. asparagus) and leaves (e.g spinach) eaten all contain tissues capable of lignification, they are consumed at a relatively immature stage of development having undergone little lignification (Selvendran 1987). The age, development and surrounding environment will dictate the shape and composition of the primary cell wall which has numerous roles in plant tissues. Size, shape, disease resistance, active defence, grafting and cell regulation are all functions ascribed to the primary cell wall (McNeil *et al* 1984). The bulk of the biomass of plants is their cell walls and this is the major source of DF in animal nutrition. Before considering and understanding the role of DF in the diet, the structure and function of primary cell walls is reviewed. For further information, the reader is referred to McNeil *et al.* (1984) and Fry (1988).

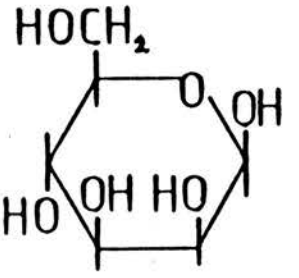
Primary cell walls are composed of approximately 90% polysaccharide and up to 10% protein (as glycoprotein). The polysaccharides can be divided into several classes: cellulose, hemicelluloses and pectins. The other polymer groups in the cell wall are glycoproteins (e.g. extensin) and lignin. The major monomeric



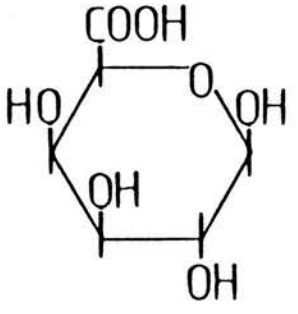
D-Glucose



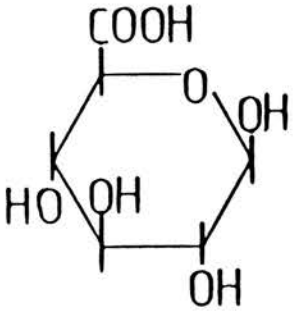
D-Galactose



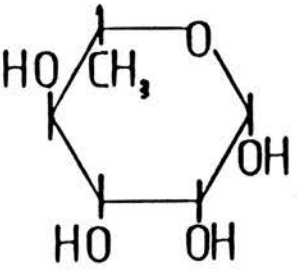
D-Mannose



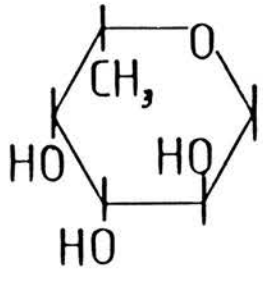
D-Galacturonic acid



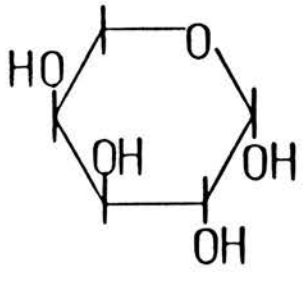
D-Glucuronic acid



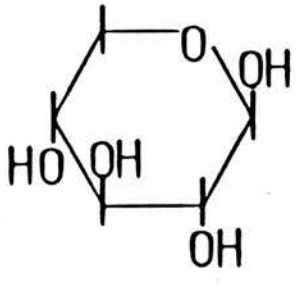
L-Rhamnose



L-Fucose



L-Arabinose



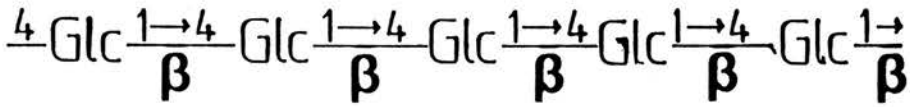
D-Xylose

Figure 1.1 : Chemical structures of the common monosaccharides found in the primary plant cell wall.

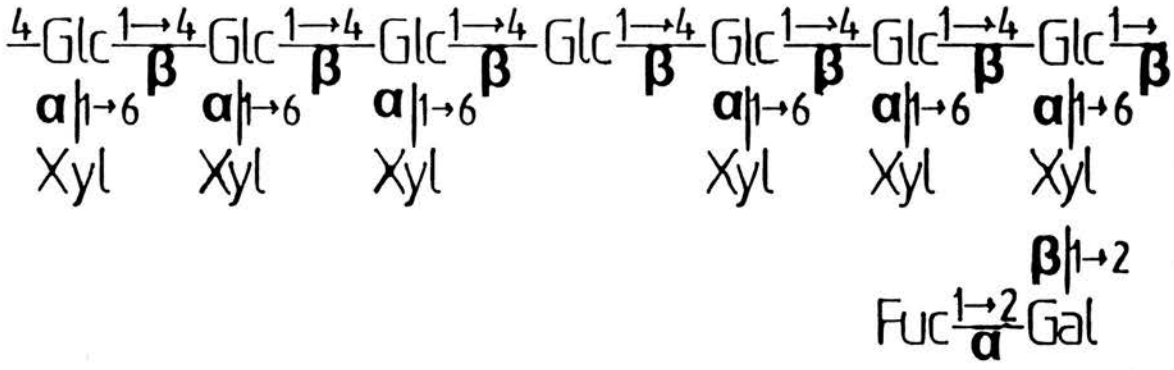
constituents of the polysaccharides are given in Fig. 1.1. Prior to the formation of the polymers, the sugars are activated by transfer to a nucleoside diphosphate (NDP) group producing an NDP-sugar (Fry 1985). The polymers are synthesised on membranes (cellulose on the plasmalemma and the pectins and most hemicelluloses on the endoplasmic reticulum and/or the golgi bodies) which catalyze the addition of sugar residues from NDP-sugars to the growing polysaccharide chain. It is unclear whether or not there are other intermediary carriers (e.g. glycoproteins or glycolipids) to transfer these sugar residues (Green & Northcote 1979). It is thought that polysaccharide synthesis is controlled by NDP-sugar availability and/or the activity of the polysaccharide synthase enzymes. As mentioned earlier, there are several classes of polysaccharide in the primary cell wall:

Cellulose

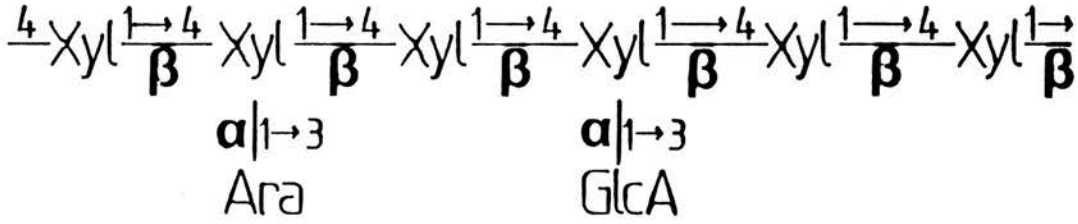
This is the polysaccharide most associated with cell walls of plants and is most abundant in the secondary cell walls. It is an unbranched $\beta(1\rightarrow4)$ linked D-glucose chain (Fig. 1.2) which can aggregate with other chains to form microfibrils. The microfibrils can either be in a highly ordered crystalline conformation or a less ordered amorphous conformation containing other wall polysaccharide. Essentially cellulose is a structural component in the primary cell wall although there is a correlation between the orientation of the new microfibrils and the principle axis of growth (Preston 1974).



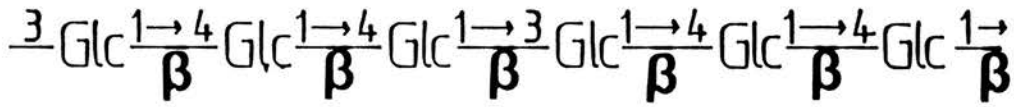
Cellulose



Xyloglucan



Xylans



β-Glucans

Figure 1.2 : Structural composition of cellulose and hemicelluloses found in the primary plant cell wall.

Hemicelluloses

Xyloglucans (XG): A major hemicellulose in the cell walls of dicots occurring in lesser quantities in monocots and irrespective of the source these polysaccharides are structurally similar. They have a cellulose-like backbone of β(1→4) linked D-glucose with side chains on about 75% of these residues (Fig. 1.2). The side chains occur in different ratios depending on the source but appear to have a definite order. The majority of XGs are hydrogen bonded to the cellulose but a small amount may also be associated with pectic polysaccharide, proteins and polyphenolics (Selvendran 1985). Although of major structural importance to the cell, XG may also have a regulatory role in the cell wall. The nonasaccharide XG9, liberated from xyloglucan by the action of cellulase, exhibits anti-auxin activity in pea stem segments (McDougall & Fry 1988).

Xylans: These polysaccharides are the major hemicellulose in the primary cell walls of monocots and are less common in those of dicots. They contain a backbone composed of β(1→4) linked D-xylose residues (Fig. 1.2) which can hydrogen bond, like XG, to cellulose in a strong association. The xylose residues may carry L-arabinose and/or D-glucuronic acid side groups and the extent of this substitution will determine the solubility and hydrogen bonding capacity of the xylan (McNeil *et al.* 1975). Specific short oligosaccharides have been reported attached to the arabinose side groups. Many of the xylose residues are O-acetylated, about 50% of the glucuronic acid residues are ether-linked to methyl groups and a few of the terminal arabinose residues carry feruloyl esters. These feruloyl esters may undergo peroxidase-catalyzed coupling with other xylan side groups (Markwalder & Neukom 1976) and these cross-links may have important effects on the

physical properties of the cell wall.

β-Glucans: These polysaccharides are found in greater abundance in monocots than dicots. They are essentially unbranched β(1→4) and β(1→3) linked chains of D-glucose residues (Fig. 1.2). The β(1→3) linkage accounts for about 30% of the polysaccharide and the others are β(1→4). The occurrence of long stretches of β(1→4) region may enable the polysaccharide to hydrogen bond to cellulose.

Pectins

The pectins have distinct regions with respect to polysaccharide composition which may be either a single polysaccharide with different regions or different polysaccharides covalently attached to each other. The precise functions of pectins in the cell wall are unclear although the pectic polysaccharides of the middle lamella are thought to act as a biological glue, through ionic bonds, cementing cells together (Selvendran 1985). They occur in higher amounts in the primary than secondary cell walls which indicates a possible role in growth. Whatever function is ascribed to these polysaccharides in dicots an equivalent will have to be found in monocots which contain small quantities of pectic polysaccharides.

Homogalacturonans: They consist mainly of α(1→4) linked D-galacturonic acid residues with occasional α(1→2) linked L-rhamnose (Fig. 1.3). The galacturonic acid-rich regions can become cross-linked by calcium bridges, whereas those residues which are methylesterified are unable to undergo cross-linking. It is thought that the occa sional rhamnose residue in the backbone may give rise to irregular structures and flexibility in the chain (Rees & Wright 1971).

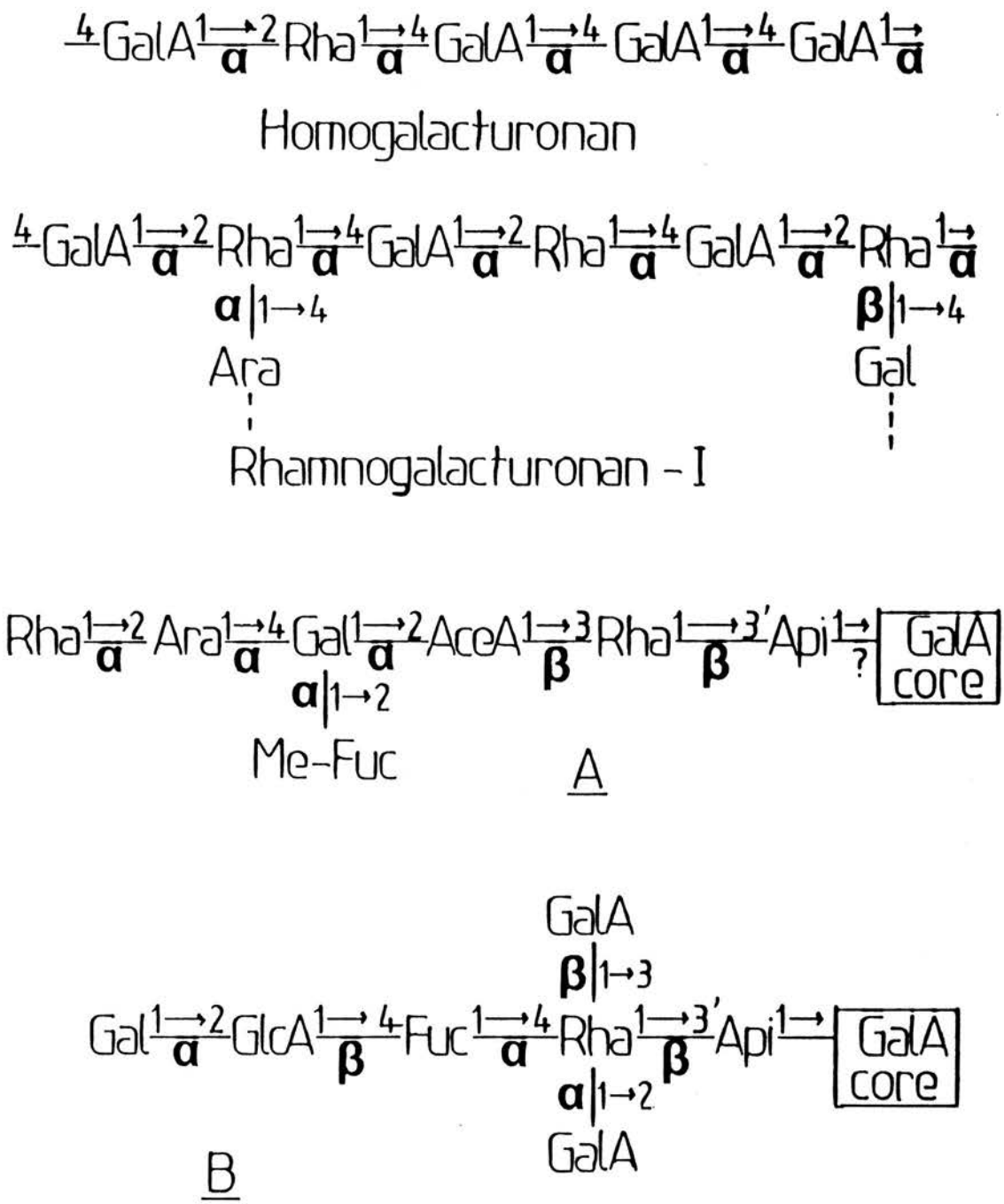


Figure 1.3 : Structural composition of the pectic polysaccharides of the primary plant cell wall.

Rhamnogalacturonan I (RG-I): This pectic polysaccharide has a repeating disaccharide unit of α(1→2) linked L-rhamnose and α(1→4) linked galacturonic acid (Fig. 1.3). Ap proximately 50% of the rhamnose residues carry side groups of differing length and complexity although D-galactose and L-arabinose are the most common sugars in the side chains. The galacturonic acid residues in the backbone may also be methylesterified.

Rhamnogalacturonan II (RG-II): It is structurally very different from RG-I especially in its side group composition. There appears to be a galacturonic acid-rich core with several side chains in the form of two different heptasaccharides (Fig. 1.3 - A and B) and some smaller side groups (Melton *et al.* 1986). Although there appears to be a defined number of side chains per RG-II molecule it is not known whether these are attached to the backbone in a particular sequence.

Glycoproteins

There are numerous enzymes associated with the primary cell wall each with a specific and individual function. In general they are glycoproteins but every enzyme is different and detailed analysis of their carbohydrate composition is difficult.

Extensins: As their name suggests, these glycoproteins were originally thought to be involved in cell extension, although recently it has been proposed that extensin is necessary for the formation of a proper cell wall (Cooper 1984). They are present in varying amounts in dicots and monocots but are abundant in the walls of cultured cells. Extensins are basic glycoprotein with a polypeptide backbone rich in hydroxyproline. The hydroxyproline residues frequently carry short oligosaccharide side groups of α(1→3) and β(1→2) linked arabinose

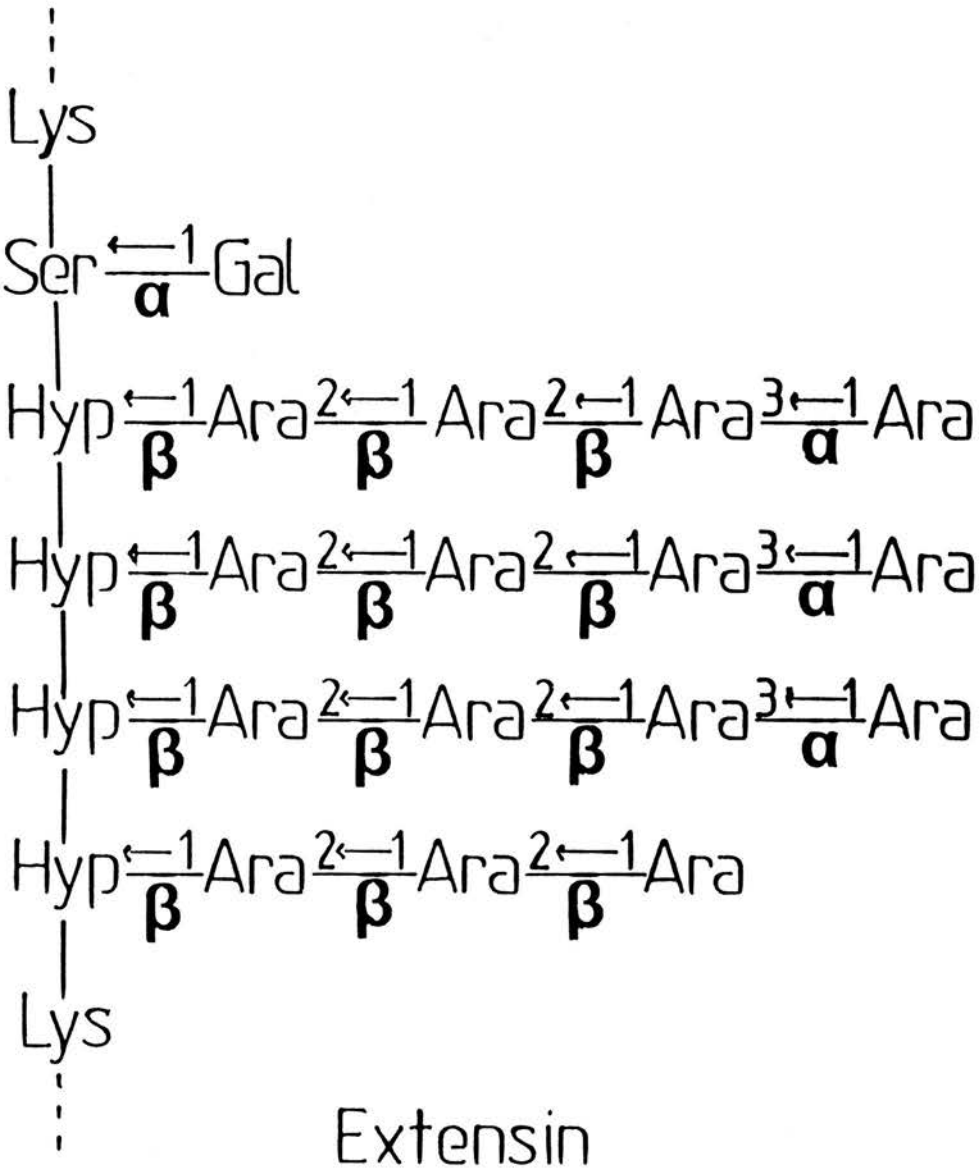
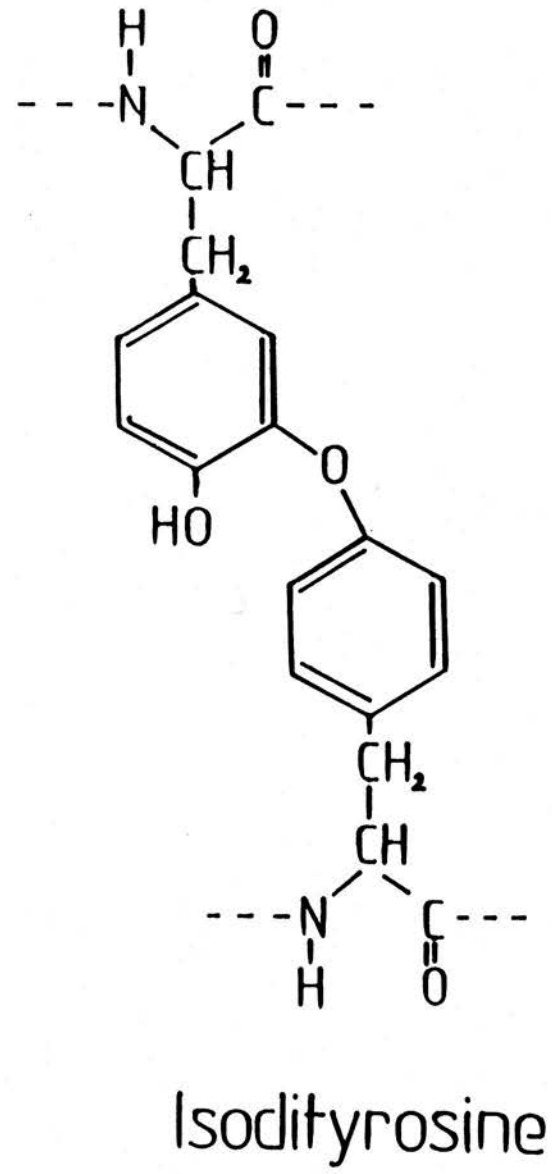


Figure 1.4 : Structure of the extensin polypeptide backbone with the oligosaccharide side-chains.

(Fig. 1.4). Some of the serine residues may also carry α-D-galactose groups. The carbohydrate component of extensin contributes about 50% of the weight of this glycoprotein, the rest comes from the polypeptide. Perhaps its most remarkable feature is its insolubility in normal protein solvents- boiling SDS, phenol/acetic acid/ water (2:1:1) and cold acid or alkali. The reason behind this insolubility is the formation of isodityrosine cross-linkages (Fig. 1.5). These form between two tyrosine residues which are oxidatively coupled by wall bound peroxidase (Fry 1982b). Recently Lamport and Epstein (1983) introduced their "warp-weft" hypothesis as a model for the 3-dimensional structure of the plant cell wall. It suggests that the cellulose microfibrils and extensins form a network surrounded by a pectin/hemicellulose gel. This they argue will enable the stresses of the cell wall to be distributed evenly throughout the microfibrillar network. For a more in-depth look at the role of extensin in the cell wall see Wilson and Fry (1986).

Arabinogalactan proteins (AGP): These are structurally similar to extensins in that they are rich in hydroxyproline residues which may or may not carry short oligosaccharide arabinose side chains. However the polypeptide backbone accounts for only 2-10% of the weight and most of the hydroxyproline residues possess long polysaccharide chains attached through β-D-galactose residues. The rest of the polysaccharide chain is complex and the other principal sugars in the chain are arabinose (furanose and pyranose forms), glucuronic acid and galactose. The precise role of AGPs is unclear although lubrication and cell recognition have been suggested.



Isodityrosine

Figure 1.5 : Structure of the isodityrosine cross-link which forms between two tyrosine residues in the extensin backbone.

The overall view of primary plant cell walls is of a complex, three dimensional network of microfibrils which has many properties and functions. With this in mind it is difficult to believe that the polysaccharide fraction of the plant cell wall is the only part to remain intact after passage through the G.I. tract. It is far more probable that the entire cell wall reaches the caecum/colon and is a fermentable substrate for the bacterial flora. The consequence of this would mean that the glycoproteins in the cell wall would have to be considered as part of the DF definiton as well as the polysaccharides and lignin. This is discussed in more detail in chapter 5.

Dietary Fibre in Health and Disease

From the above description of plant cell walls it is apparent that DF is a heterogeneous group of fibres which differ greatly in their properties. It is perhaps this non-uniformity that has made the investigation of DF in health and disease so confusing. It was Surgeon-Captain T.L. Cleave who stimulated interest in DF by suggesting that refined foods led to overconsumption which further led to diseases found in Western civilisations (Cleave 1974). Although his writings were based primarily on the harmful effects of refined sugar cane there was some emphasis on the possible protective action of fibre. This theme was explored by Painter who was responsible for the treatment of diverticular disease by reducing intracolonic pressure with bran (Painter 1975). The aetiology of many other Western diseases has been attributed to a reduced fibre intake: varicose veins, deep venous thrombosis and haemorrhoids (Burkitt 1972), appendicitis, constipation and colo-rectal cancer (Burkitt 1973),

diabetes mellitus (Trowell 1975), obesity, coronary thrombosis, gallstones, duodenal ulcer, hiatus hernia, and constipation (Trowell and Burkitt 1987).

There have been many studies investigating the possible beneficial effects of certain fibre sources (Cummings *et al.* 1978, McLean Ross *et al.* 1983, Judd and Truswell 1985 and Eastwood *et al.* 1986). However, the many different fibre preparations used have very different effects in the G.I. tract and the contribution of each different cell wall polysaccharide in the whole diet is impossible to determine. As DF passes through the G.I. tract it remains relatively undigested until it reaches the caecum or colon. It is in these areas, in non-ruminants, that fermentation of the DF takes place by the bacterial microflora. The properties of the different DF components will affect their mode of action in the G.I. tract. DF solubility and viscosity may affect the absorption of nutrients through the mucosal wall (Eastwood and Brydon 1985). However Edwards *et al.* (1987) failed to show differences between the *in vitro* viscosity of different polysaccharides and their ability to reduce the postprandial blood glucose. They suggest that a better correlation is achieved after acidification and reneutralisation of the viscous polysaccharides, to mimic the effects of passage through the upper G.I. tract.

The effect of DF on stool weight may be an important factor although the mechanism behind this is not straight-forward. Stephen and Cummings (1979) have shown an inverse relationship between water holding capacity of fruit and vegetable fibre and stool weight and this may be due to the extent of fermentation of the DF in the caecum. The fermentation of DF may influence stool weight by increasing bacterial proliferation and, hence, the bacterial concentration in the

stool. The bacterial contribution to the stool is also affected by whole gut transit time (Stephen *et al.* 1987), which can be influenced by DF levels in the diet. However, these changes in transit time were achieved by using drugs (Senokot, codeine and loperamide) and hence other pharmacological effects may be involved. The suggestion that bacterial growth and water holding capacity are the only effectors of faecal mass and transit time has been questioned by Tomlin and Read (1988). They have reproduced the effect of wheat bran on stool weight, transit time and stool frequency by using inert plastic pellets. These findings may have resulted from the mechanical abrasion and stimulation of mucosal receptors to induce secretions (increase faecal weight) and stimulate peristalsis (increase transit time). The relationship between the physical properties of individual fibres and their physiological effects in the G.I. tract is a complex one, which requires more fundamental research.

Bacterial Fermentation

The caecum is, perhaps, the most complicated area of the G.I. tract and it is in this region that bacterial activity is at its greatest. The caecum should, in some ways, be considered as an organ in its own right because of the complex interaction between bacterial species and the host. Fermentation is the process by which anaerobic bacteria breakdown organic components to yield energy for growth and maintenance (Singleton and Sainsbury 1978). The fermentation pathways of these saccharolytic bacteria are very complicated and involve many different species interactions (Miller and Wolin 1979, Wolin and Miller 1983). The major products of bacterial fermentations are short chain fatty acids (SCFAs), methane, hydrogen and carbon dioxide. The

pathways for the production of the major SCFAs can be seen in Figure 1.6. The SCFAs produced are quantitatively the principal metabolites and the importance of bacterial fermentation to the host is the fate of these SCFAs in the body (Cummings and Englyst 1987). SCFAs instilled into the colon of humans are efficiently absorbed and metabolised by the host and this is thought to represent a significant calorific salvage from an otherwise unavailable source (Høverstad *et al.* 1982).

The individual SCFAs produced have been implicated as energy sources in different host tissues and organs. Roediger (1982) demonstrated that isolated colonic epithelial cells could utilise SCFAs and that butyrate was the preferred fuel. Sakata (1987) has shown an increase in gut epithelial cell proliferation in the presence of *n*-butyric acid which does not occur with other SCFAs (e.g. acetate or propionate). Cellular studies have shown that butyrate has antineoplastic properties which may be important in the health of the colon with respect to colo-rectal cancers (Kruh 1982).

The other major SCFAs produced (acetate and propionate) will pass into the portal vein after absorption and then into the liver. In the liver propionate is thought to be involved in gluconeogenesis and may mediate the hypocholesterolaemic effect of certain fibres (Chen *et al.* 1984). The only SCFA to be found after passage through the liver is acetate and it is thought to be an important metabolic precursor in muscle tissues (Knowles *et al.* 1974). Although it is known that SCFAs are absorbed through the colonic mucosa, the mechanism is not clear.

It is thought that the majority of the SCFAs pass through the colonic mucosa as the un-ionised acid by passive diffusion although this alone would be too slow to explain the rapid movement of

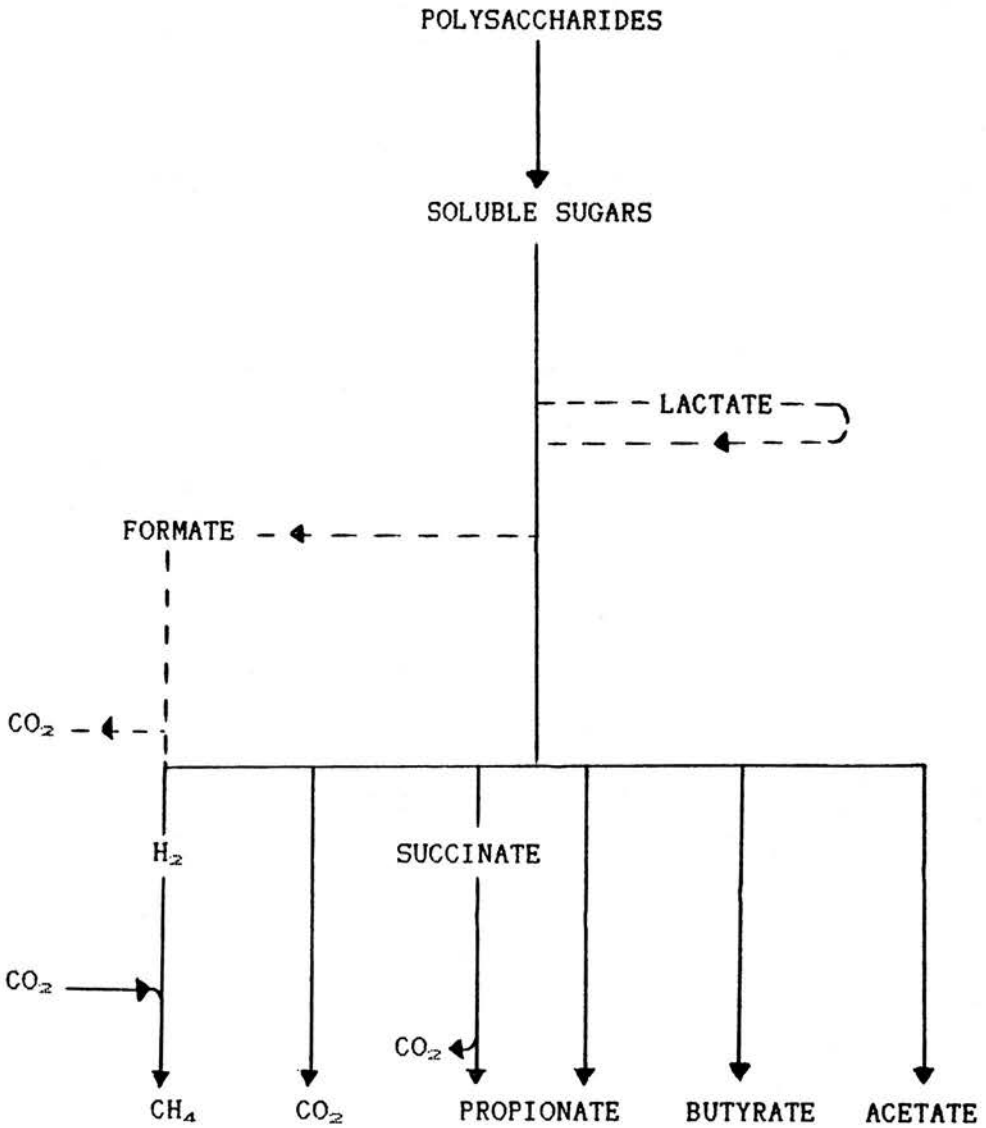


Figure 1.6 : Fermentation pathway of dietary polysaccharides in the rumen. Dashed lines represent minor pathways.

(Adapted from Wolin and Miller 1983)

these acids (Cummings and Branch 1984). The absorption of SCFAs is characterised by the accumulation of bicarbonate in the lumen and a stimulation of sodium absorption (Roediger and Moore 1981), possibly by the recycling of hydrogen ions after dissociation from the acid in the mucosal cells (Von Engelhardt and Rechkemmer 1983). Although the exact mechanism is not clear, the principal effect of SCFA absorption from the colon is to produce a net movement of water out of the colonic lumen by the absorption of sodium (Cummings and Branch 1984).

The human caecum is virtually inaccessible for sampling and an alternative model for study was required. *In vitro* fermentations using a faecal inoculum have been widely used to predict the events which may occur in the caecum/colon (Tomlin *et al.* 1986, Englyst *et al.* 1987a, McBurney and Thompson 1987). A possible problem is that although faecal material may possess similar bacterial populations these bacteria may have different activities to those in the caecum. Other workers have taken the caeca of freshly killed animals to determine the fermentation activities of the bacteria (Goering and Van Soest 1979, Bonhomme-Florentin 1988). Englyst *et al.* (1987a) have shown that different polysaccharide sources will be fermented at different rates and they postulate that, in the G.I. tract, there may be a hierarchy of polysaccharides utilised by bacteria. For each polysaccharide fermented, there was a different final molar ratio of SCFAs produced, suggesting that different pathways and, posssibly, different bacterial populations were used in the fermentations. However these studies should be treated with caution as the *in vitro* environment is not identical to the caecum/colon with respect to absorption of metabolites, secretion of bicarbonate, concentrations of bile salts, etc (Salyers 1984).

Using faecal bacterial populations to determine the caecal/colon activities in humans is virtually unavoidable, but it is felt that the faecal population is close to the caecal/colonic populations and is therefore a reasonable replacement. However how can the use of bacterial populations from the caeca of freshly killed rats be useful in the investigation of human colonic function ? The problem of extrapolating from the bacterial activities in the rat caecum to the events of the large bowel in man have been investigated. Van Soest *et al.* (1983) compared the fermentation of DF in man and with other animals and found that it was more extensive than in the rat but less than in swine. More recently, Nyman *et al.* (1986) carried out a variety of feeding trials in both rats and man and concluded that "the rat experimental model is useful for the prediction of fermentative break down and bulking capacity of dietary fibre in man". Although the use of rats to investigate the fate of DF has its drawbacks, it is a useful model for detailed analysis of the ^{14}C distribution in host tissues after fermentation of ^{14}C-plant cell walls in the G.I. tract.

The Use of Radioactivity in Dietary Fibre Studies

One of the earliest studies to use radioactivity in feeding trials investigated the fate of fermentation products in the caecum of rats and humans (Bond and Levitt 1976). They showed that [^{14}C]acetic acid instilled into the caecum was converted into $^{14}CO_2$ quickly irrespective of a bacterial presence. However [^{14}C]glucose was not converted to $^{14}CO_2$ when instilled into the caecum of animals treated with antibiotics. This emphasises the need for a gut microflora to scavenge unabsorbed carbohydrate and produce SCFAs which can be utilised by the host. Despite these initial findings, there is little

work on radioactive components of DF and their fate in the G.I. tract. However, two groups have recently been trying to evaluate the calorific salvage that occurs in the caecum/colon of man after ingestion of [U-^{14}C]lactitol (Grimble *et al.* 1988), an artificial sweetener, and [U-^{14}C]fructooligosaccharides (Hosoya *et al.* 1988), both of which are undigested in the small intestines. Both groups reported considerable fermentation by the bacteria to produce ^{14}C-SCFAs available for absorption from the colon and utilisation by the host.

Cellulose break down in man has been investigated using both ^{131}I (Carryer *et al.* 1982) and ^{14}C (Kelleher *et al.* 1984, Walters *et al.* 1989) labelled cellulose, although contamination of the cellulose preparations was a problem in assessing its break down. These studies differed in the extent of fermentation of the labelled cellulose which was also seen in non-radioactive studies (Prynne and Southgate 1979, Van Soest *et al.* 1982). Walters *et al.* (1989) showed that an increase in the fibre intake (ispaghula) resulted in a rise in the level of $^{14}CO_2$ produced from the [^{14}C]cellulose preparation which was probably due to the increased bacterial mass. All three radioactive tracer studies were carried out in human subjects and hence the amount of information about the fate of the metabolites in the tissues was limited.

Advantages and Aims of The Project

This introduction has highlighted the areas of current interest in the fermentation of DF in the caecum and colon. Clearly there are limitations with the methodology available and interpretation of results obtained is far from conclusive. There appears to be a difference between the fermentation of different fibre

sources as exemplified by the cellulose degradation results (Cummings 1984). It is not clear whether isolated polysaccharides behave in the same manner as chemically identical wall bound polysaccharides. The recent work that has been carried out to investigate the role of certain polysaccharides in our diet has relied on either *in vitro* fermentations or, indirectly, using ileostomy patients or measuring expired gases as a marker of colonic fermentation. There is obviously a limit to the amount of relevant information which these experiments can yield.

Because plant cell walls are the major source of DF in our diets, the use of U-^{14}C-labelled plant cell walls to study DF metabolism has important advantages. The radioactive label will enable the passage of the ^{14}C-plant cell walls through the G.I. tract, the incorporation of the ^{14}C-labelled fermentation products into host tissues and the evolution of $^{14}CO_2$ to be followed. The plant cell walls contain many polysaccharide types (pectin, cellulose and hemicelluloses) in their natural state. This probably reflects, more closely, the presentation of these polysaccharides to the bacterial flora in the G.I. tract and hence reflects the mechanism of DF metabolism. The host diet can be altered to examine the effect of a high/low fibre diet on the different bacterial populations and their capacity to ferment the ^{14}C-plant cell walls.

The aims of this thesis were to develop an experimental procedure to investigate the break down of a radioactive dietary fibre marker in the G.I. tract and to monitor the fate of the fermentation products. The growth of plant cell cultures in radioactive medium and isolation of their cell walls has been established in Fry's laboratory. However, the extensive analysis of the ^{14}C distribution in

the cell walls required prior to the animal studies was not available and had to be developed. The animal studies planned were designed to answer specific questions regarding the fermentation of the ^{14}C-plant cell walls in the rat G.I. tract.

1) To what extent is this ^{14}C dietary fibre marker broken down ?

2) Assuming that there is some fermentation by the bacterial flora, what is the fate of the products ?

3) Are different polysaccharides of the same tissue type degraded to the same extent in intact plant cell walls ?

4) Where is the ^{14}C distributed after passage of ^{14}C-plant cell walls through the rat G.I.tract ?

Using *in vitro* fermentations, the caecal bacterial activity can be investigated in isolation from the rest of the animal model. This would yield information regarding the production of ^{14}C-SCFA and the bacterial contribution to $^{14}CO_2$ production. The *in vitro* and *in vivo* studies together will provide a window in which to view the caecal activity regarding DF fermentation.

The final aim of this project was to compare the effect of a high and low fibre diet on the break down of the DF marker. It is thought that the low fibre fed animals would have a reduced capacity to ferment DF although the reduced fibre intake would slow transit time and the bacteria would have longer to ferment the fibre. It is not apparent what effect the high and low fibre diets would have on the hosts ability to utilise any ^{14}C-SCFAs produced.

CHAPTER 2 : MATERIALS AND METHODS

MATERIALS

Chemicals

All chemicals used were obtained from Sigma Chemical Company or BDH Ltd. and were of AnalaR quality when available. The Distilled water used was singly distilled in a glass still.

Radiochemicals

The D-[U-^{14}C]glucose used to label the spinach cell suspensions was obtained from Amersham International plc. (specific activity 10 GBq/mmol).

Equipment

All the paper for paper chromatography was supplied by Whatman International Ltd. High voltage paper electrophoresis (HVPE) was carried out on a flatbed, water-cooled system from Shandon with a power pack from Savant Instruments Incorporated. A radioactivity-TLC-analyser from Raytest Instruments Ltd. was used to scan paper chromatograms.

Determination of the ^{14}C content of samples was carried out by complete oxidation in a Packard 306 sample oxidiser obtained from Packard Instruments Ltd. Scintillation counting was carried out in an Intertechnique SL3000 from Kontron Instruments.

Samples were dried under vacuum in a Speed-vac centrifugal evaporator from Savant Instruments Incorporated. Centrifugation was carried out in a Centaur 2 bench-top centrifuge from M.S.E. Suspensions cultures were disrupted in a Sonirep 150 sonicator from

Short-chain fatty acids were separated on a Gas liquid chromatogram obtained from Carlo Erba. Freeze-drying of faeces, G. I. contents and tissues was carried out in a freeze-dryer from ChemLab Instruments Ltd.

Cell Suspension Cultures

Cell suspension cultures of spinach (*Spinacia oleracea* L., cv Monstrous Viroflay) were grown in 1 l flasks containing 400 ml of a defined medium (Appendix; Table 1) and incubated on an illuminated orbital shaker (diam. of orbit = 1.3 cm) at a speed of 90 rev/min and at a temperature of 25°C. The cells were maintained by the aseptic transfer of 4-5 g fresh weight of cells into 400 ml of fresh medium every 2 weeks. All flasks were sealed with sterile cotton wool bungs and covered in aluminium foil to prevent contamination.

Cell Wall Isolation

The cell suspension cultures were harvested 2 weeks after subculture by filtration through nylon mesh. The following procedure was used to isolate plant cell walls free from contamination by other cell components. After washing with distilled water, the cells were resuspended in 1 l of a solution containing 25 mM $LaCl_3$ and 10 mM ascorbic acid and the pH adjusted to 4.4 with 1 M NaOH. The cells were agitated for 5 min to remove any ionically bound proteins, filtered through nylon mesh (64 µm pore diameter), rinsed in distilled water and frozen in 10 g portions. Each portion was thawed and suspended in 100 ml of a 2% (w/v) SDS solution containing 20 mM HEPES and 10 mM ascorbic acid and the pH adjusted to 7.4 with 1 M NaOH. The cell suspensions were sonicated 4-5 times for not more than 1 min and cooled between each sonication. A sample of each suspension was examined under a light microscope to ensure cell disruption was complete. Each suspension was filtered through nylon mesh, washed in

the HEPES buffer and plunged into 100 ml of phenol/acetic acid/water (PAW 2:1:1 w/v/v).

At this stage, the portions were pooled and the PAW suspension was stirred for 16 h at 20°C in a fume cupboard. The PAW suspension was filtered through muslin and the presence of protein in the filtrate determined by addition of 0.05 ml of 10% (w/v) ammonium formate and 5 ml acetone to 1 ml of the PAW filtrate. The solution was mixed and left to stand on ice for 1 h, during which time any protein that was present formed a white precipitate. The PAW treatment of the cell wall residue was repeated several times until the protein precipitation test proved negative. The cell wall residue was then washed 4-5 times in distilled water to remove any traces of the PAW and weighed (X g) before adding to 9 x X ml of dimethyl sulphoxide (DMSO). The DMSO treatment was required to remove any starch which was present in the cell wall preparation. The cell wall suspension was left to stir for 16 h at 20°C in a fume cupboard, filtered on nylon mesh, washed 4-5 times in distilled water and dialyzed against distilled water for 16 h to remove any trace of the DMSO. At the end of this treatment the cell walls were freeze-dried.

U-^{14}C-Labelled Plant Cell Walls

The medium used to produce U-^{14}C- labelled plant cell walls was the same as outlined in the appendix (Table 1) for the growth of plant cell cultures except that the glucose concentration was reduced from 1% to 0.5% to encourage uptake of the ^{14}C. Each 1 l flask contained 400 ml of medium, 1 mCi of D-[U-^{14}C]glucose and 4-5 g fresh weight of cells. The flasks were incubated as outlined earlier until the activity of the medium had decreased to less than 20% of the

original activity (i.e. until more than 80% of the [^{14}C]glucose had been taken up by the cells). The cell walls were then isolated by the procedure outlined above.

Purification of Driselase

Driselase is a collection of enzymes produced from the fungus *Irpex lacteus* and before use, it was purified. 15 g of the crude mixture was dissolved in 150 ml of distilled water, stirred gently and cetrifuged at 4000 rpm for 5 min to remove any debris. The supernatant was removed and the pellet washed with 50 ml of distilled water, recentrifuged and the supernatants pooled. The final volume was made up to 200 ml and 105 g of ammonium sulphate was added to the supernatant slowly, with gentle stirring. This solution was stored in the fridge for 16 h and then centrifuged at 4000 rpm for 10-15 min. The clear supernatant was discarded and the pellet washed in an ammonium sulphate solution. After centrifugation at 4000 rpm, the supernatant was discarded and the pellet resuspended in 100 ml of distilled water. This solution was run through a Bio-gel P-2 column with distilled water as the eluant. The colourless eluate was discarded and the first 125 ml of coloured eluate was collected and freeze-dried.

Driselase Hydrolysis

The enzymic digestion of freeze-dried cell walls, faeces and G.I. contents was achieved using purified Driselase. The buffer used for the hydrolysis was distilled water containing 1% (v/v) pyridine, 1% (v/v) acetic acid and 0.05% (w/v) sodium azide. The purified Driselase was added at a final concentration of 1% and the ratio of

Driselase solution: freeze-dried material was 1 ml: 1 mg. The hydrolysis was carried out in Pyrex tubes with teflon sealed caps in a shaking incubator at 37°C for 16h.

Acid Hydrolysis

There were two different types of acid hydrolysis using either trifluroacetic acid (TFA) or hydrochloric acid (HCl):

TFA hydrolysis- to release the monosaccharides from the polysaccharides of the cell wall. 1 ml of 2M TFA was added to 1 mg of the cell wall material in a glass tube which was then sealed and incubated at 120°C for 1 h. The tube was centrifuged to remove any debris and the supernatant dried under vacuum in the Speed-vac evaporator to remove the TFA.

HCl hydrolysis- to release amino acids from proteins by cleaving the peptide bonds. 1 ml of 6 M HCl containing 10 mM phenol was added to 3-5 mg dry weight of sample in a Pyrex tube which was then sealed with a Teflon cap and incubated in an oven at 110°C for 16 h. The debris was removed by centrifugation and the supernatant dried under vacuum in the Speed-vac evaporator to remove the HCl.

Paper Chromatography

Descending paper chromatography was used with Whatman 3MM chromatography paper and two different solvents:

butan-1-ol/acetic acid /water (BAW 12: 3: 5 v/v/v)

ethyl acetate/pyridine/water (EPW 8: 2: 1 v/v/v)

The chromatography was conducted in special chromatography tanks contained within a fume cupboard for 16 h. However for some

samples, using the EPW solvent, longer periods were required to separate certain monosaccharides which ran close together e.g. D-glucose and D-galactose.

Paper Electrophoresis

Uronic acids were separated by paper electrophoresis on Whatman 3MM paper. The solvent system used was:
acetic acid/pyridine/water (APW 10:1:189 v/v/v), pH 3.5, 2kV for 3 h and picric acid as a marker.

Radioactivity -TLC- Analyser (RITA)

To determine the position of the radioactivity on either paper chromatographs or paper electrophoretographs, the RITA was used. The detector head of the RITA is lowered onto a 20 cm sample lane on the paper. Radiation particles are released from the sample and cause localised ionization of the gaseous mixture (10% methane/90% argon v/v) in the detector head. A strong electric field, generated by a high voltage wire, converts the signal into electrical charge by secondary ionization. The electrical signal passes along the wire in both directions from the site of initial ionization. The arrival time of the charge at either end of the detector head machine is used to determine the position of the radioactivity in the sample lanes. A typical RITA scan can be seen in chapter 3 (Fig. 3.1).

Stains

To visualise the marker and sample lanes on both paper chromatograms and paper electrophoretograms, the following stain was used for monosaccharides: a solution containing 1.6% (w/v) phthalic

acid in a mixture of 490ml acetone, 490ml diethyl ether and 20ml water was used. Immediately before use, 0.5ml of aniline was added to 100 ml of this solution. The paper was then dipped in this solution, dried and heated in an oven at 105°C for 5-10 min. The pentose sugars stain red/brown, the hexose sugars stain dark brown and the uronic acids stain orange.

Scintillation Counting

Triton scintillant- Aqueous samples were analysed for their radioactive content using this scintillation cocktail with 0.33% (w/v) PPO and 0.033% (w/v) POPOP in toluene/Triton X-100 (2:1 v/v). The sample: scintillant ratio was 1:10 (v/v) in a final volume of 11 ml.

Non-Triton scintillant- Solid samples (e.g. chromatography paper) were analysed in small strips using this scintillation cocktail containing 0.5% (w/v) PPO and 0.05% (w/v) POPOP in toluene. The amount of scintillant required was dependent on the size of the piece of chromatography paper (0.5 ml of scintillant for a 2 x 1 cm strip).

Animals

The animals used in all of this work were male albino Wistar rats which were bred within the Animal Unit, Western General Hospital, Edinburgh. Unless otherwise stated, the rats were housed in solid-bottomed cages with wood shavings for bedding and the room was regulated to a 12 h light/dark régime. The temperature was maintained at 20°C and the noise level was kept to a minimum. The stock diet, CRM(X) (Appendix; Table 2), and water were supplied *ad libitum*. Some animals were weaned onto a low fibre diet but were otherwise maintained in the same manner as the CRM(X) animals. In the appendix;

Table 3 there is a comparison between the high fibre [CRM(X)] diet and the low fibre diet.

Dosing of Radioactivity

The animals were dosed with a known amount of U-^{14}C-labelled plant cell walls suspended in water, by gavage (volume not exceeding 3 ml). This technique delivers the radioactivity directly into the rats stomachs reducing errors at dosing. The administering of the U-^{14}C-plant cell walls to the rats occurred at 10.00 a.m. in every experiment to eliminate the effects of diurnal variation. The syringe barrels were washed out and the residual activity determined by sample oxidation (see Sample Oxidation below), providing an accurate measure of the ^{14}C dose.

Animal Experimentation

After dosing with the U-^{14}C-plant cell walls the animals were placed into individual cages of two different types:

metabolic cages- these cages were used in experiments where the animals were housed for more than 24 h. The broad-spaced gridded floor prevents the rats from performing coprophagy and enabled the faeces and urine to be collected. In these studies, the faeces and urine were collected into vials containing 0.1 M NaOH to prevent further bacterial activity and every 24 h the samples were removed and frozen at -20°C.

metabolism/respiration cages- (Fig. 2.1) these cages were used for short-term experiments in which the expired gases could easily be collected. The pattern of $^{14}CO_2$ production was followed by removal of

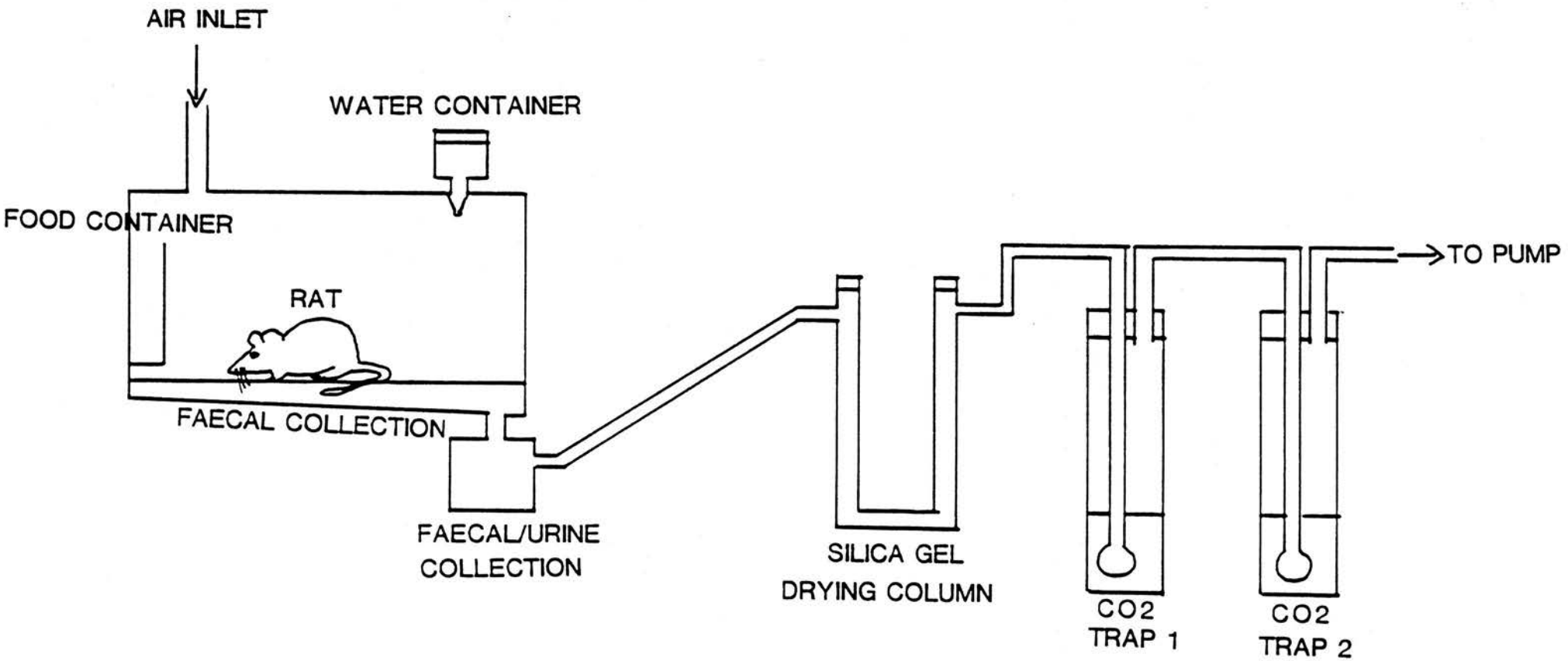


Figure 2.1.: Metabolism/respiration cage and apparatus for the detection of $^{14}CO_2$ (See text for description).

a 1 ml sample of Carbosorb from the CO_2 trap, addition of 7 ml of fresh Carbosorb and 10 ml of Permafluor and counting of the radioactivity in a scintillation counter. These cages had the disadvantages that they could not separate the faeces and urine as effectively as the metabolic cages. The faeces and urine were not collected directly into NaOH as this would absorb some of the expired CO_2. At the end of the experiment, 0.1 M NaOH was added to both the faeces and urine before freezing.

Post Mortem and Tissue Handling

On the conclusion of the experiments, the animals were killed by cervical dislocation and dissected immediately. The small intestines, caecum and colon were isolated and removed and their contents transferred to pre-weighed vials before freeze-drying. The intestinal tissues were washed thoroughly in distilled water and frozen. The other tissues removed for analysis; adipose tissue, liver and skin, were frozen immediately after dissection. As well as the G.I. contents, the faeces, urine and liver tissues were also freeze-dried before analysis.

Sample Oxidation

Weighed aliquots of each sample, <0.2 g, were oxidised in a stream of oxygen using a Packard 306 sample oxidiser. The carbon dioxide produced was collected in Carbosorb/Permafluor (4:5 v/v) and the ^{14}C content determined by liquid scintillation counting.

Fermentations

The fermentations were carried out in 100 ml Quickfit wide-

mouth flasks containing 40 ml of the medium in a shaking water bath at 37°C. The medium was the same as that used by Goering and Van Soest (1979) and the main constituents are listed in the appendix (Table 4). The flasks were fitted with air-tight rubber stoppers with three openings: a gas inlet tube, a gas outlet tube (connected to a CO_2 trap) and a culture sample tube. After addition of the medium to the flasks containing 4 g of plant cell walls and a known amount of ^{14}C-labelled plant cell walls, the flasks were assembled in a water bath. The air inlet tube was connected to a gas cylinder containing 5% carbon dioxide and 95% nitrogen which was pumped through the flasks. The reducing solution was added after 30-40 min through the culture sample tube and the flasks were left until the medium was completely reduced, as determined by a change of colour in the medium from red (oxidised) to colourless (reduced).

For each flask, one rat was killed by cervical dislocation, its caecum was isolated at the terminal ileum and proximal colon using surgical thread and then it was dissected out. When the colour change was reached, the rat caecum was opened and the contents added directly to the flask. The flasks were incubated for 24 h and culture samples removed at 3, 6, 9, 12 and 24 h. To each sample was added a drop of 1 M NaOH to bring the pH back to neutral (the short chain fatty acids produced are volatile when acidic), before freezing. The production of $^{14}CO_2$ was monitored in the same way as described in the animal experiments.

Short Chain Fatty Acid Analysis

The analysis of short chain fatty acids (SCFAs) was carried out using the method of Spiller *et al.* (1980). A solution containing

1% of each of acetic, propionic, iso-butyric, butyric, iso-valeric and valeric acids, the pH adjusted to 7 with 1 M NaOH, was used to construct a standard curve for each SCFA. Different volumes of the stock solution were used (10-200 µl) and the volume made up to 0.8 ml with distilled water. To these standards were added 50 µl of β-methylvaleric acid (as an internal standard) and 100 µl of orthophosphoric acid (converts the SCFAs into their acidic form). The SCFAs were extracted from the aqueous phase with 3 x 3 ml diethyl ether and the ether fractions pooled for analysis. The SCFAs in the ether fraction were separated using a gas chromatogram with column packing Chromosorb W-AW 80/100 mesh coated with 10% SP 1200 and 1% H_3PO_4 and detected by flame ionisation. The temperature programme was 80°C to 150°C at 16°C per min and the flow rate of the carrier gas (oxygen-free nitrogen) was 40 ml per min.

By plotting the peak height ratio of SCFA: internal standard against the concentration of each SCFA in the standard, a calibration curve was constructed for the individual SCFAs (Fig. 2.2 and 2.3). For analysis of SCFAs from the fermenter, 0.8 ml of culture was added to 50 µl of internal standard and 100 µl of orthophosphoric acid. The samples were extracted with ether and analysed as described above. By using the standard curves obtained, the level of each SCFA in the samples was determined.

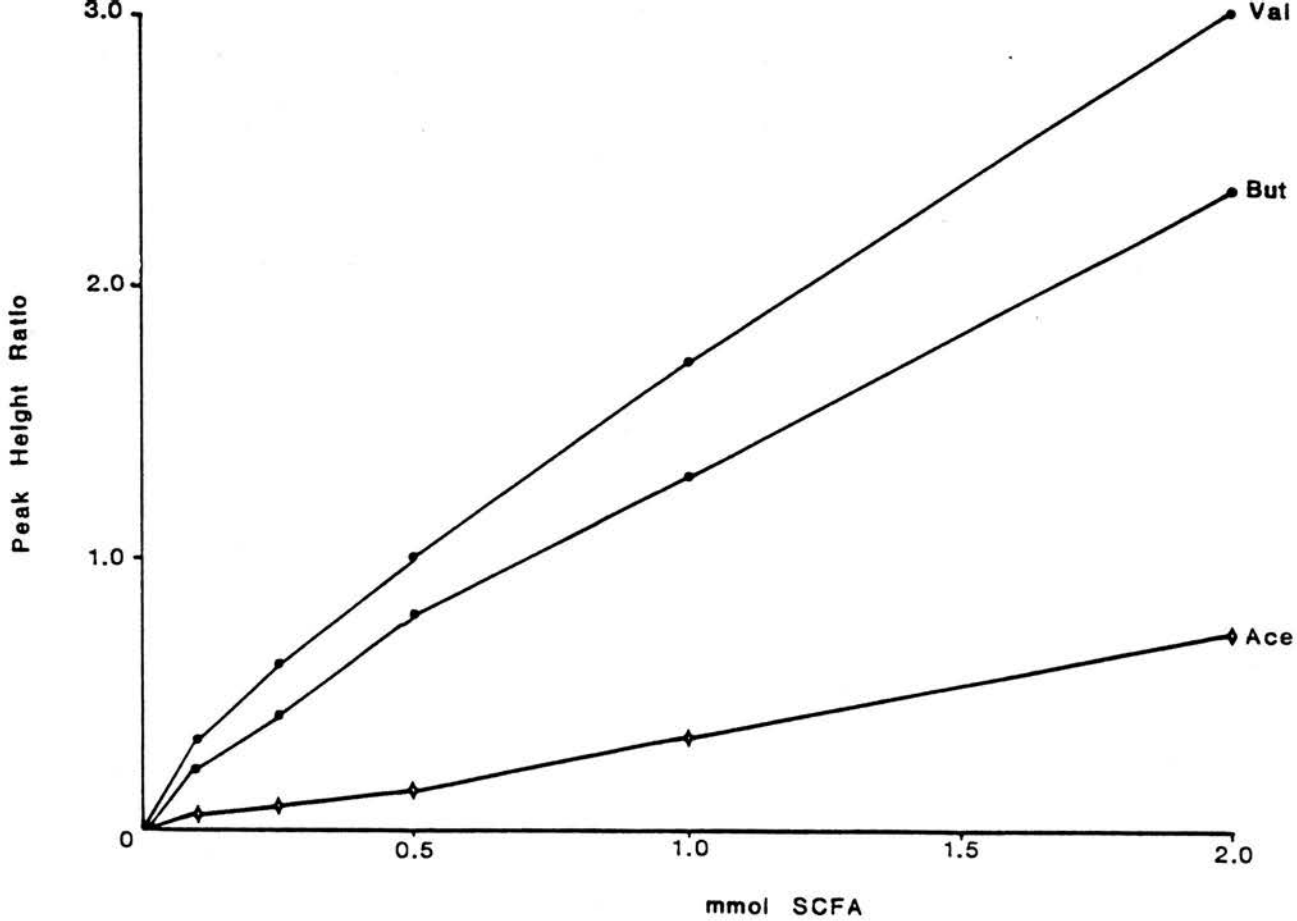


Figure 2.2.: Standard curves for the determination of the levels of valerate (Val), butyrate (But) and acetate (Ace) in fermentation samples.

$$\text{Peak height ratio} = \frac{\text{peak height of SCFA}}{\text{peak height of internal standard}}$$

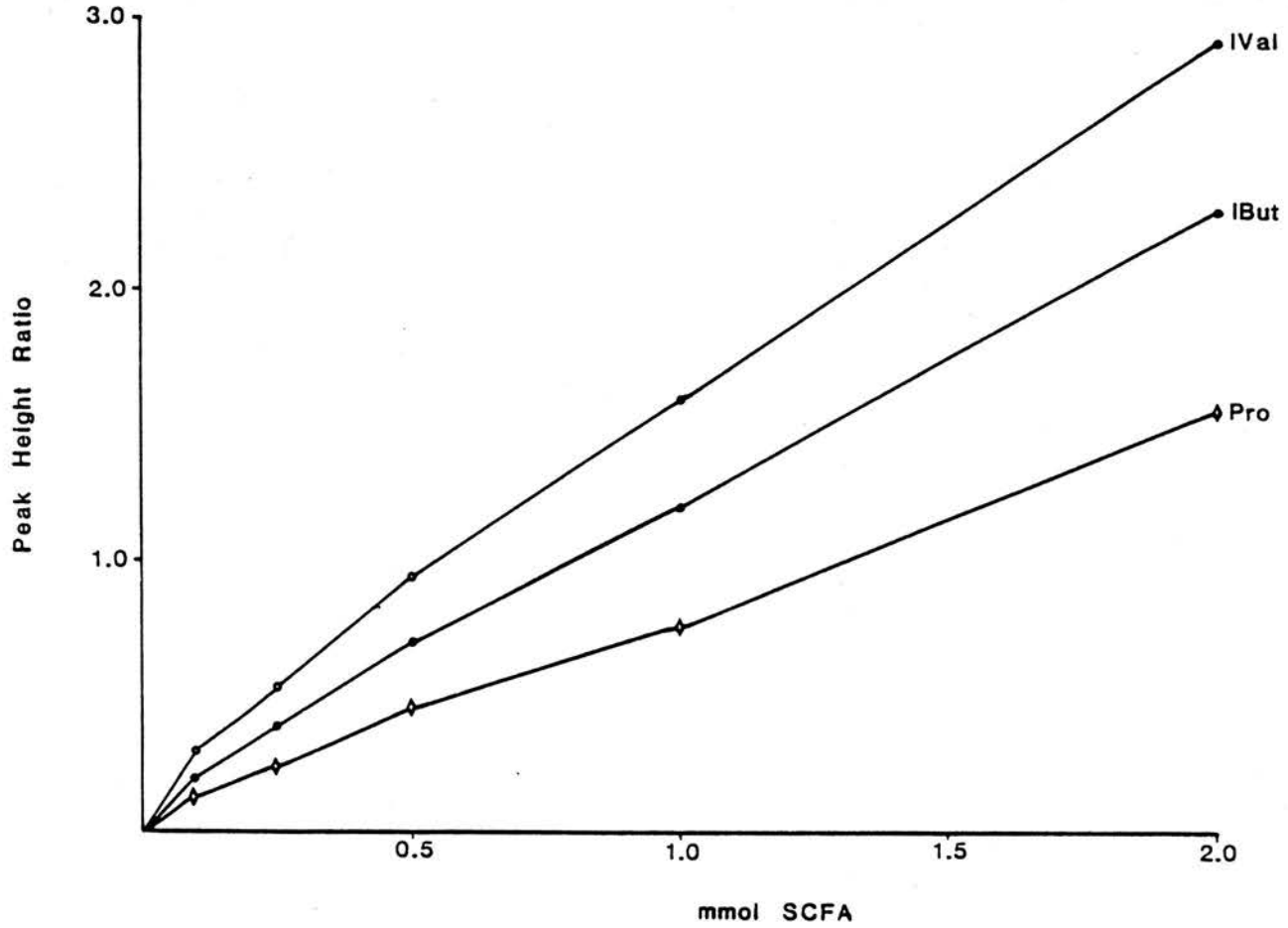


Figure 2.3.: Standard curves for the determination of the levels of iso-valerate (IVal), iso-butyrate (IBut) and propionate (Pro) in fermentation samples.

$$\text{Peak height ratio} = \frac{\text{peak height of SCFA}}{\text{peak height of internal standard}}$$

CHAPTER 3 : RESULTS I

ISOLATION AND CHARACTERISATION OF U-^{14}C-LABELLED PLANT CELL WALLS

Aims

The aim of this section of work was to produce a U-^{14}C-labelled plant cell wall preparation from cultured spinach cells. The ^{14}C-labelled plant cell walls were analysed using various chemical and enzymic treatments to determine the distribution of the ^{14}C.

Introduction

By using plant cell cultures as the source of DF it was relatively simple to manipulate the medium and introduce a radioactive label. *In vivo* feeding studies using different radioactive sugars has revealed that specific sugar residues of wall polysaccharides become labelled (Fry 1988). Because glucose is a central metabolite, the addition of [U-^{14}C]glucose to the medium will result in the uniform labelling of the cell wall with ^{14}C. However, the plant cells will also use the [^{14}C]glucose as an energy source and label other organic components of the cells (e.g. proteins, storage compounds, genetic material, etc.).

Using non-radioactive cell walls there was a higher than normal level of protein associated with the cell wall which was difficult to remove. Various techniques, both chemical and enzymic, were employed to remove the protein with differing degrees of success (e.g. hot PAW was fairly successful at removing the protein but also altered the monosaccharide profile, removing some arabinose from the cell walls). It was therefore necessary to employ a fairly rigorous isolation procedure to eliminate possible contamination from ^{14}C-compounds not normally associated with the cell wall. The protein content of the cell wall preparation was monitor ed at various stages of the isolation procedure which is outlined in the materials and

methods chapter. The major stages of the isolation procedure were sonication, repeated PAW treatments and de-starching with DMSO. The level of protein in our cell wall preparation was calculated at 3.4% of the total cell wall which was within the range expected in primary cell walls (McNeil *et al.* 1984).

During the three year project two separate ^{14}C-plant cell wall preparations were produced. Although the spinach cells were grown under the same conditions and the walls isolated using the same procedure, the distribution of the ^{14}C in the cell walls was not identical. During this chapter, reference will be made as to which ^{14}C-plant cell wall preparation was used (either I or II). In this chapter, both of the ^{14}C-plant cell wall preparations were analysed in considerable detail although ^{14}C-plant cell wall preparation I includes analysis not performed on ^{14}C-plant cell wall preparation II and these differences are clearly indicated.

Estimate of the Specific Activity of ^{14}C-Plant Cell Wall I

Immediately before the freeze-drying stage of the isolation procedure, duplicate samples of the ^{14}C-plant cell wall suspension were removed. The volumes were made up to 1 ml with distilled water and 10 ml of Triton scintillant was added. The activity of each sample was determined by assuming that the efficiency of counting aqueous samples in the scintillation counter was 70% and converting the scintillation readout (cpm) into activity (dpm). During the initial studies the activity of the cell wall preparation was based on these calculations. However in later studies a quench correction curve was constructed and used to determine the counting efficiency of each sample individually (Fig. 3.6).

After freeze-drying, the ^{14}C-plant cell walls were weighed and the activity per mg calculated as:

1.04×10^6 dpm per mg ^{14}C-plant cell walls.

Monosaccharide Analysis by TFA Hydrolysis

Trifluoroacetic acid (TFA) hydrolysis was carried out on the ^{14}C-plant cell wall preparation I which would result in the hydrolysis of most of the non-cellulosic glycosyl linkages. The undegraded, predominantly cellulosic, material was separated from the non-cellulosic supernatant by centrifugation and the two fractions were analysed.

Non-Cellulosic Fraction

The dried supernatant was resuspended in 0.5 ml of distilled water and 50 µl was paper chromatographed in EPW. After drying, the chromatogram was scanned by the RITA and the results are shown in Figs. 3.1 and 3.2.

The two RITA scans show the same chromatogram but with different y-max scales to emphasise the presence of at least four and possibly five minor peaks of ^{14}C, which co-chromatographed with the external standards shown. However the largest peak of ^{14}C remained at the origin and this was thought to be a [^{14}C]galacturonic acid peak as galacturonic acid is immobile in this solvent system (Fry 1988). However there may have been some [^{14}C]cellulose contaminating this fraction which would remain at the origin.

To remove this doubt, the origin was eluted in distilled water and dried under vacuum. The residue was hydrolysed with Driselase to break down any cellulose into glucose and 50 µl of the

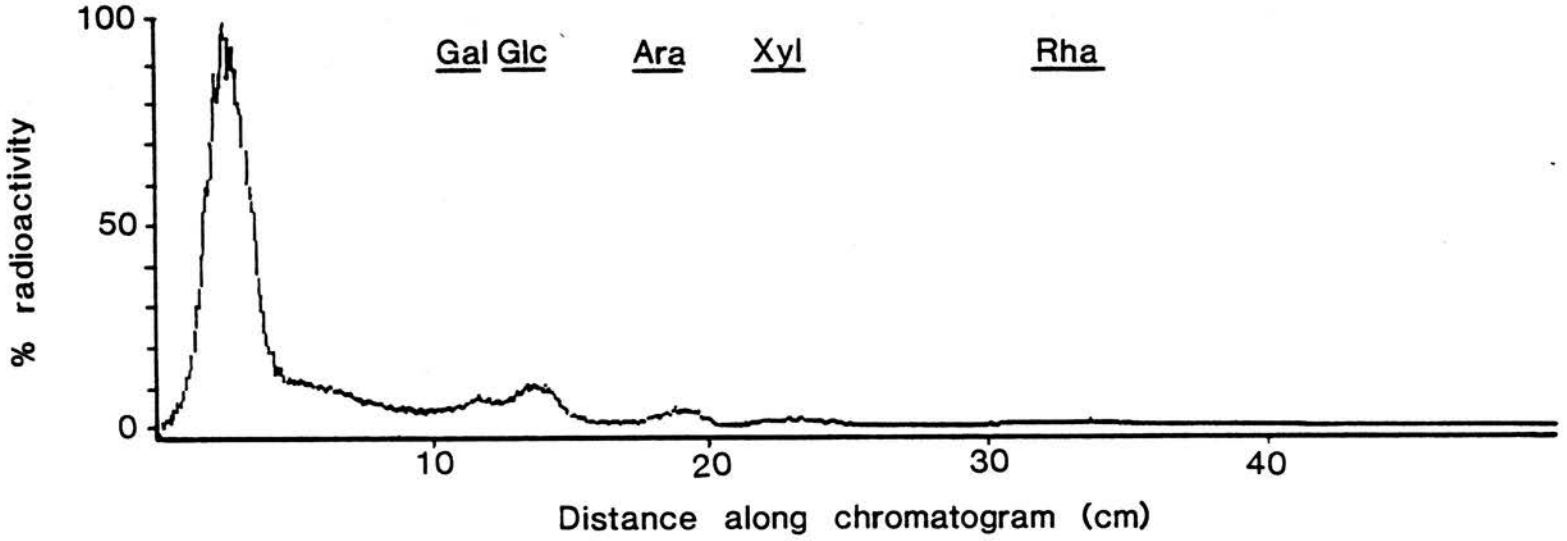


Figure 3.1 : Radioactive profile of the non-cellulosic fraction of ^{14}C-plant cell walls after development on paper chromatography in EPW for 16 h.

y-max. (100% radioactivity per channel) = 1329 counts

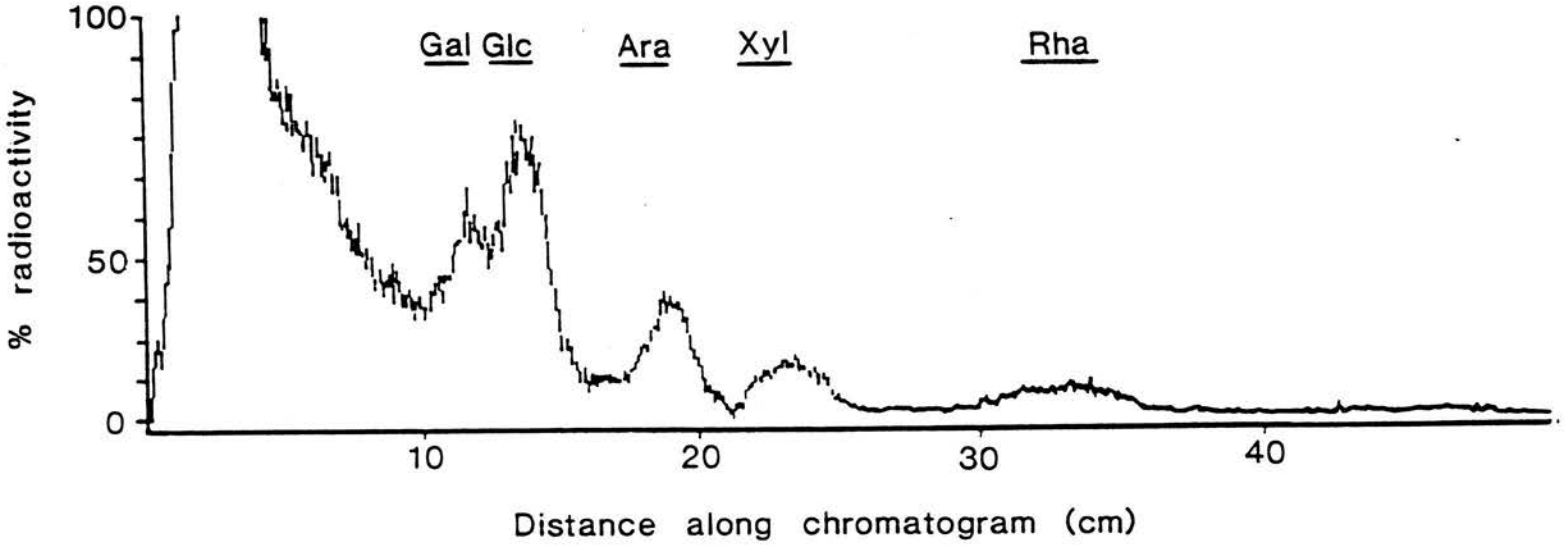


Figure 3.2 : Radioactive profile of the non-cellulosic fraction of ^{14}C-plant cell walls after development on paper chromatography in EPW for 16 h.

y-max. (100% radioactivity per channel) = 200 counts

supernatant was chromatographed in BAW for 16 h. The chromatogram was scanned by the RITA (Fig. 3.3).

The RITA scan showed only one peak of ^{14}C and this appears to co-chromatograph with the galacturonic acid external standard, although the separation of the galacturonic acid and glucose standards was poor.

To confirm the view that this peak of ^{14}C was [^{14}C]galacturonic acid, the region of interest was eluted from the chromatogram and loaded onto a paper electrophoretogram to detect uronic acids (see methods). The electrophoretogram was scanned by the RITA (Fig. 3.4).

The activity of the peak had decreased but it was still possible to detect one single ^{14}C peak which co-chromatographed with the galacturonic acid external standard.

From these three results it was clear that the non-cellulosic fraction contains mostly [^{14}C]galacturonic acid. This is only found in the pectin fraction of the primary cell wall. The other minor ^{14}C components are galactose, glucose, arabinose, xylose and rhamnose. In this analysis the RITA was used qualitatively, as a means to identify the monosaccharides present in the cell wall fractions but not to quantify them.

<u>Cellulosic Fraction</u>

After the TFA treatment of the ^{14}C-plant cell wall preparation I described above, the pellet was removed and dried under vacuum. The pellet was hydrolysed with Driselase and 50 µl of the supernatant was loaded onto a paper chromatogram. To enhance the separation of the monosaccharides, the chromatogram was run in BAW for

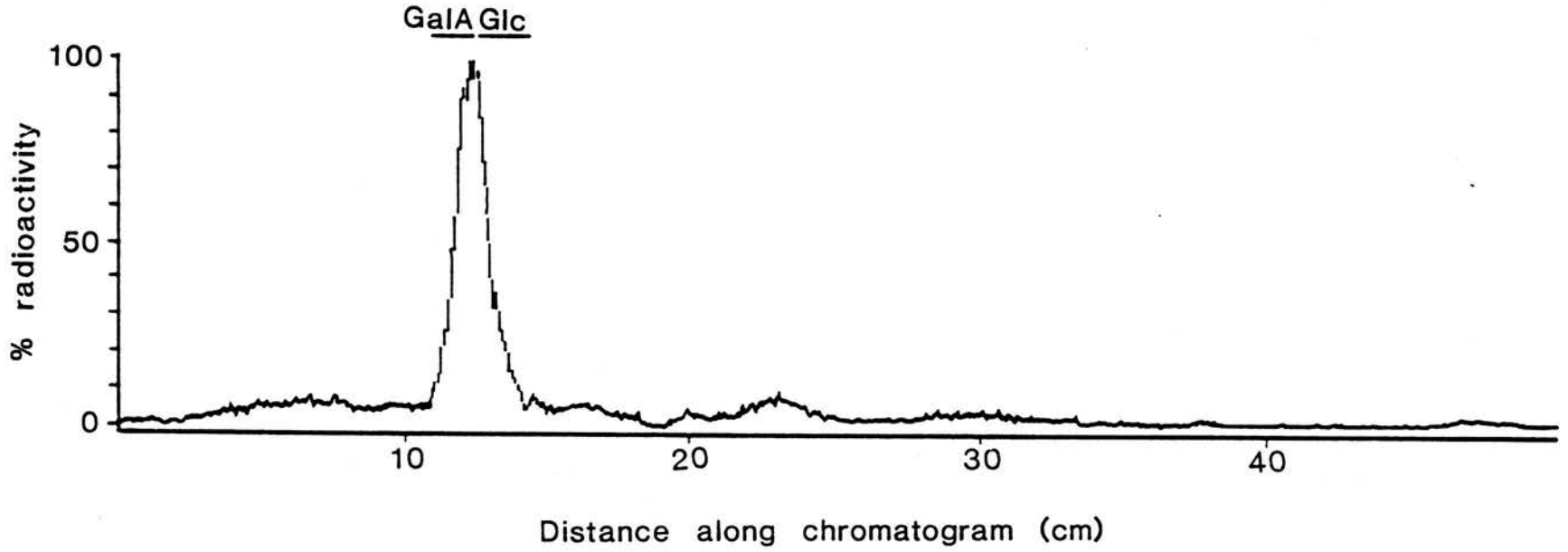


Figure 3.3 : Radioactive profile of the Driselase treated origin from Fig. 3.1 after development on paper chromatography in BAW for 16 h. y-max. (100% radioactivity per channel) = 199 counts

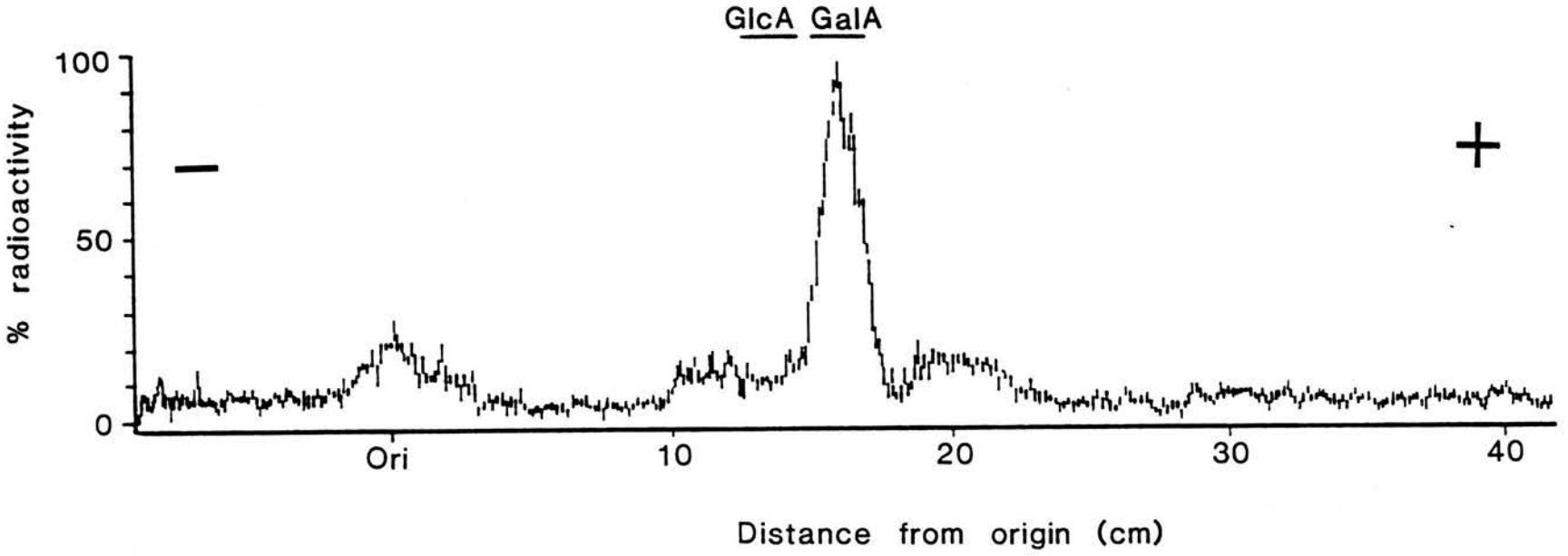


Figure 3.4 : Radioactive profile of the putative galacturonic acid region from Fig. 3.3 after development on paper electrophoresis in APW at 2 kV for 3 h.

y-max. (100% radioactivity per channel) = 29 counts

16 h followed by EPW for 16 h. The dried chromatogram was scanned by the RITA (Fig. 3.5).

The RITA scan shows one large ^{14}C peak which co-chromatographed with the glucose external standard and three other smaller peaks. The first of these small peaks remained at the origin and may well be undegraded protein or polysaccharide material. The second peak co-chromatographed with galacturonic acid and is probably the result of contamination from the supernatant after TFA hydrolysis. The third peak did not co-chromatograph with any monosaccharide standard, but it may correspond to a disaccharide. Xyloglucan is not broken down completely to monosaccharides by Driselase. The Driselase enzyme mixture does not contain any α-xylosidase to cleave the (α1→6)xylosyl-glucose bond in xyloglucan and a disaccharide (XG2) is produced. However, according to Fry (1988), the XG2 should chromatograph closer to the GalA peak. Another possible disaccharide which would chromatograph in this region is xylobiose, although Driselase would be able to hydrolyse this disaccharide to xylose. This third peak may be XG2 or xylobiose but its exact nature is unknown.

Assay of ^{14}C

To enable the conversion of the scintillation readout of aqueous samples into ^{14}C activity (dpm), a quench correction curve was constructed using D-[U-^{14}C]glucose and increasing amounts of acetone as the quench standard. The counting efficiency of each sample is related to a value (X-value) produced by the scintillation counter along with the counts per minute (cpm). [^{14}C]Glucose (220,000 dpm) was added to each of six identical scintillation vials. The sample volumes were made up to 1 ml with distilled water and 10 ml of Triton

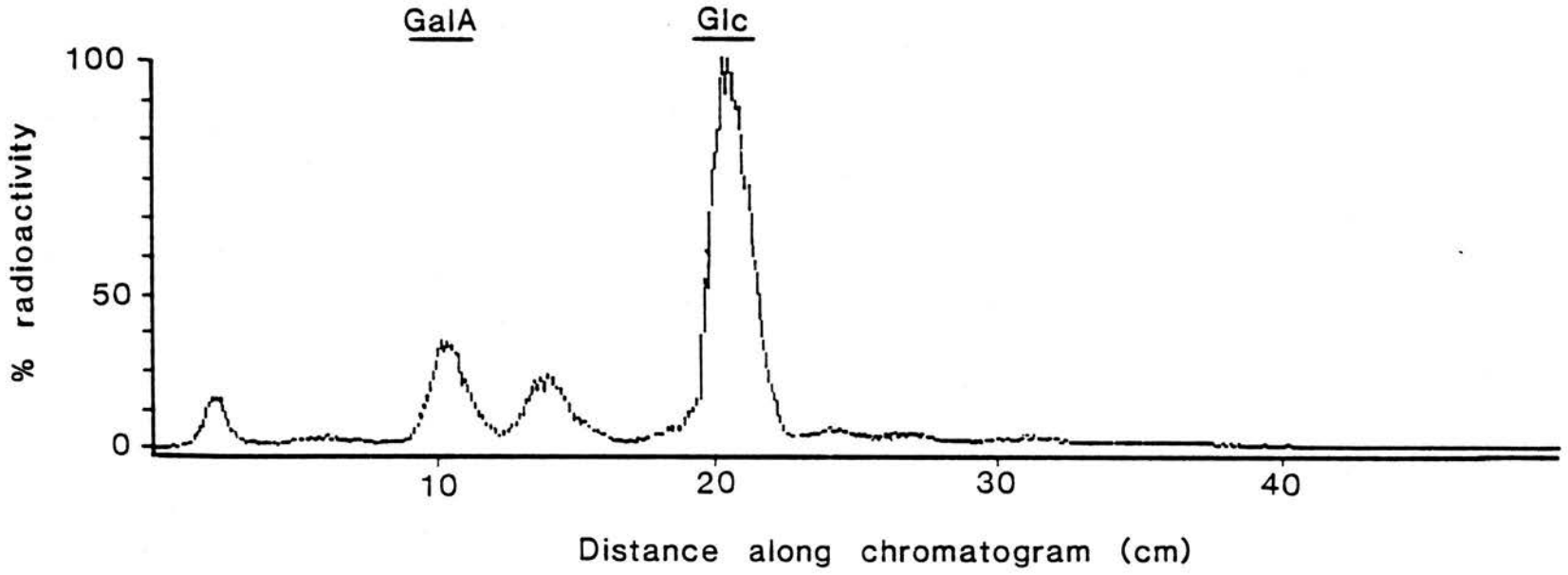


Figure 3.5 : Radioactive profile of the Driselase treated cellulosic fraction of ^{14}C-plant cell walls after development on paper chromatography in BAW for 16 h and EPW for 16 h.
y-max. (100% radioactivity per channel) = 619 counts

scintillant was added to each. The vials were mixed well and the counting efficiency and cpm determined by scintillation counting. By adding progressively more acetone to each sample, a range of counting efficiencies and X-values was obtained. Plotting the counting efficiency against the X-value, a quench correction curve was constructed (Fig. 3.6) which would enable the conversion of the scintillation readout into dpm. This curve and the scintillation conditions described were used throughout the analysis where the cpm was converted to dpm.

Quantification of ^{14}C-Plant Cell Walls

Both of the ^{14}C-plant cell wall preparations were quantified and analysed according to the methods developed during the project and outlined below. The first stage of the analysis of ^{14}C-plant cell walls was the hydrolysis of any ester-linked groups.

The importance of these linkages is not clear, although Fry (1982) has shown the presence of O-feruloyl ester groups in spinach cell cultures and suggested that they may have an important role in binding polysaccharides within the cell wall, thereby influencing cell expansion. The role of other ester groups (e.g. O-acetyl and methyl esters) is not fully understood although they may affect the three dimensional structure of the polysaccharide.

Ester-Linked Fraction (ELF)

In order to look at this fraction, 1 mg of the ^{14}C-plant cell walls were treated with 0.1 ml of 0.1 M NaOH and placed, sealed, in a shaking incubator at 25°C for 16 h. At the end of the incubation period, the reaction was neutralised by the addition of 0.1 ml of 0.2 M acetic acid and mixed thoroughly. During the 16 h

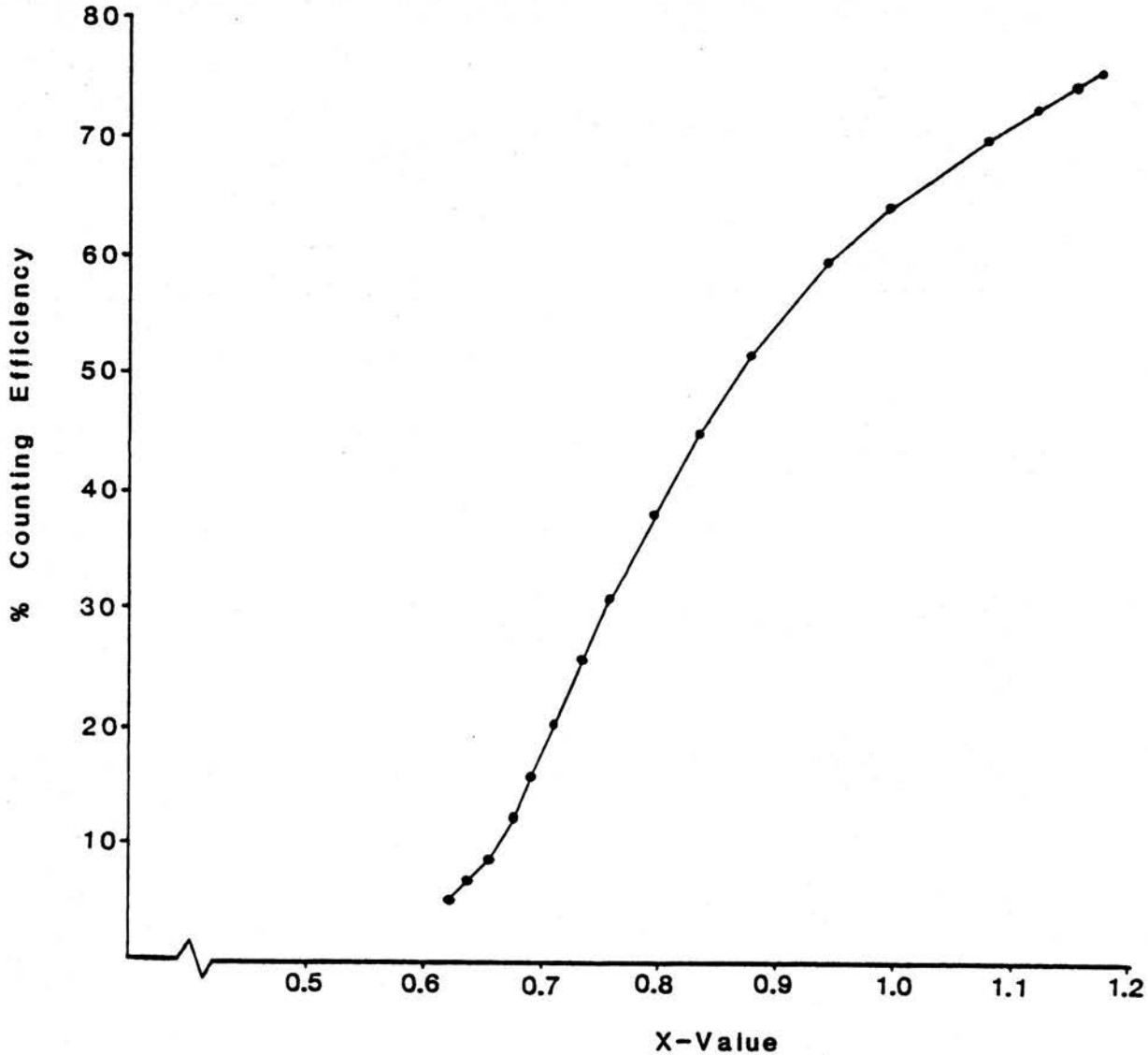


Figure 3.6 : Radioactive quench correction curve obtained using D-[U-^{14}C]glucose and acetone as the quench standard.

incubation period there may have been some solubilisation of the cell wall polymers and these were precipitated by the addition of 1 ml of absolute ethanol. After vigorous mixing, the particulate material was separated from the supernatant by centrifugation at 4000 rpm and the supernatant analysed. The ELF was divided into three aliquots to determine the level of O-[^{14}C]acetyl and [^{14}C]methyl groups.

a) The first aliquot was made up to 1 ml with distilled water and 10 ml of Triton scintillant was added before determining the activity by scintillation counting. From this value the total activity of the ELF was calculated.

b) The second aliquot was made alkaline by the addition of 0.1 ml of 2 M NaOH which would convert the [^{14}C]acetic acid to sodium [^{14}C]acetate. The solution was dried under vacuum which would result in the loss of the [^{14}C]methanol but not the sodium [^{14}C]acetate which is a non-volatile salt. The residue was redissolved in 0.5 ml of 83% (v/v) ethanol and the volume made up to 1 ml with distilled water. To this was added 10 ml of Triton scintillant before determining the activity by scintillation counting. From this value the total activity minus the [^{14}C]methanol in the ELF could be calculated and hence, the [^{14}C]methanol value. This would represent the activity of the [^{14}C]methyl ester groups in the intact cell wall.

c) The third aliquot was made acidic by the addition of 0.1 ml of glacial acetic acid which would convert any [^{14}C]acetate into [^{14}C]acetic acid. The volume was dried under vacuum which would result in the loss of any [^{14}C]acetic acid and [^{14}C]methanol. The residue was redissolved in 0.5 ml of 83% (v/v) ethanol and the volume made up to 1 ml. To this was added 10 ml of Triton scintillant before determining the activity by scintillation counting. From this value the total

activity minus the [^{14}C]methanol and [^{14}C]acetic acid could be calculated and hence the [^{14}C]acetic acid value. This would represent the O-[^{14}C]acetyl groups in the intact cell wall.

The following results were calculated from at least six mesurements in each treatment for both of the ^{14}C-plant cell wall preparations and are quoted as the mean activity (dpm/mg ^{14}C-plant cell walls) ± standard error of the mean (s.e.m.).

Table 3.1: Distribution of ^{14}C in the ELF

Fraction	^{14}C-plant cell wall preparation I (dpm/mg cell walls)	^{14}C-plant cell wall preparation II (dpm/mg cell walls)
Total	5.31 ± 0.58 × 10^4	3.04 ± 0.13 × 10^4
[^{14}C]methanol	0.84 ± 0.07 × 10^4	1.46 ± 0.09 × 10^4
[^{14}C]acetic acid	2.60 ± 0.06 × 10^4	0.65 ± 0.09 × 10^4
Other†	1.87 × 10^4	0.93 × 10^4

† Will include ester-linked phenolics

The [^{14}C]methanol fraction is representative of the ^{14}C occurring as [^{14}C]methyl ester groups in the intact cell wall. These groups are present in the pectic fraction of the cell wall attached to the galacturonic acid residues of the pectin backbone (Selvendran 1985). The [^{14}C]acetic acid fraction is indicative of the ^{14}C occurring as O-[^{14}C]acetyl groups in the intact cell wall. These groups are found predominantly in the hemicellulosic fraction of the intact cell wall attached to galactose residues of xyloglucan and xylose residues of xylans. The remainder of the ^{14}C in the ELF, not accounted for in the [^{14}C]methanol fraction or the [^{14}C]acetic acid fraction, will be other ester-linked groups released from the cell wall by the alkali treatment, including ferulate, p-coumarate and diferulate.

Driselase Soluble Fraction (DSF)

The pellet remaining after removal of the ELF was dried under vacuum before Driselase hydrolysis. Driselase is a commercially available mixture of enzymes from the fungus *Irpex lacteus* and Fry's laboratory has catalogued a substantial list of the enzymes present in this mixture. Previous work has shown that Driselase will break down greater than 98% of labelled cell wall material to its respective monosaccharides and XG2 (Fry, personal communication). Driselase will not hydrolyse the xyloglucan polysaccharide to its monosaccharides completely and the disaccharide (XG2) produced is a useful indicator of the abundance of this polymer. The monosaccharide profile after Driselase hydrolysis, therefore, is an important indicator of the polysaccharide composition of the intact ^{14}C-plant cell walls.

After Driselase hydrolysis, the suspensions were centrifuged at 4000 rpm to separate the pellet from the supernatant and the supernatant was analysed. To determine the total activity of the DSF a small sample was removed and its volume made up to 1 ml with distilled water. Triton scintillant (10 ml) was added and the activity determined by scintillation counting. The following results for the DSF in each of the two ^{14}C-plant cell wall preparations were calculated from at least 9 measurements and are quoted as the mean activity (dpm/mg ^{14}C-plant cell walls) ± s.e.m.

Table 3.2: Distribution of ^{14}C in the DSF

Sample	Specific Activity (dpm/mg cell walls)
^{14}C-plant cell wall prep. I	$5.80 \pm 0.50 \times 10^5$
^{14}C-plant cell wall prep. II	$5.39 \pm 0.06 \times 10^5$

The activity of the DSF was similar in both cases, but this does not reveal any information on the [^{14}C]monosaccharide profile in this fraction. A sample of the DSF was loaded onto a paper chromatogram and resolved in BAW for 16 h. The ^{14}C distribution along the chromatogram, in the first ^{14}C-plant cell wall preparation, was established using the RITA (Fig. 3.7) before running the chromatogram in the second solvent (EPW) for 16 h. There are no external standards in figure 3.7 because the chromatogram was not stained until after the second solvent. The chromatograms of both ^{14}C-plant cell wall preparations were scanned by the RITA after running in EPW and the results can be seen in Figs. 3.8 and 3.9.

By comparing Figs. 3.7 and 3.8, the movement of the ^{14}C peaks in the two solvents can be seen. After the BAW run, most of the ^{14}C moves away from the origin, although some remains and is probably undegraded protein and polysaccharide material. In the second solvent system (EPW), the monosaccharides separate into individual peaks which are fairly well resolved. By running external markers on the same chromatograms it is possible to relate the different peaks to specific monosaccharides. In the first ^{14}C-plant cell wall preparation (Fig. 3.8) the major peaks are galacturonic acid and glucose, although there is also a large peak at the origin. The minor peaks co-chromatograph with the galactose, arabinose and rhamnose external standards. There is also a shoulder visible on the right side of the galacturonic acid peak and it is in this region that the XG2 would run. The radioactive profile of the second ^{14}C-plant cell wall preparation (Fig. 3.9) shows a large galacturonic acid peak similar to the first preparation but the glucose peak is much smaller. There is both a galactose and arabinose peak but the rhamnose external standard ran off the

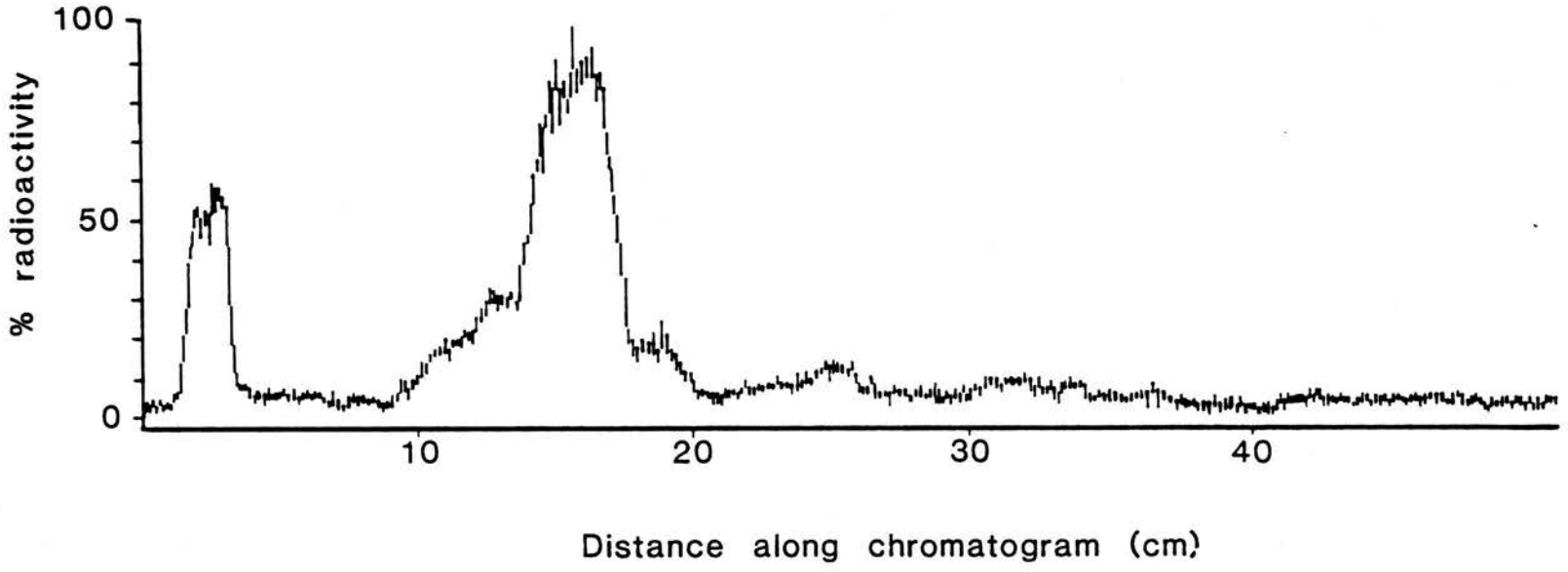


Figure 3.7 : Radioactive profile of the DSF of ^{14}C-plant cell wall preparation I after development on paper chromatography in BAW for 16 h.

y-max. (100% radioactivity per channel) = 57 counts

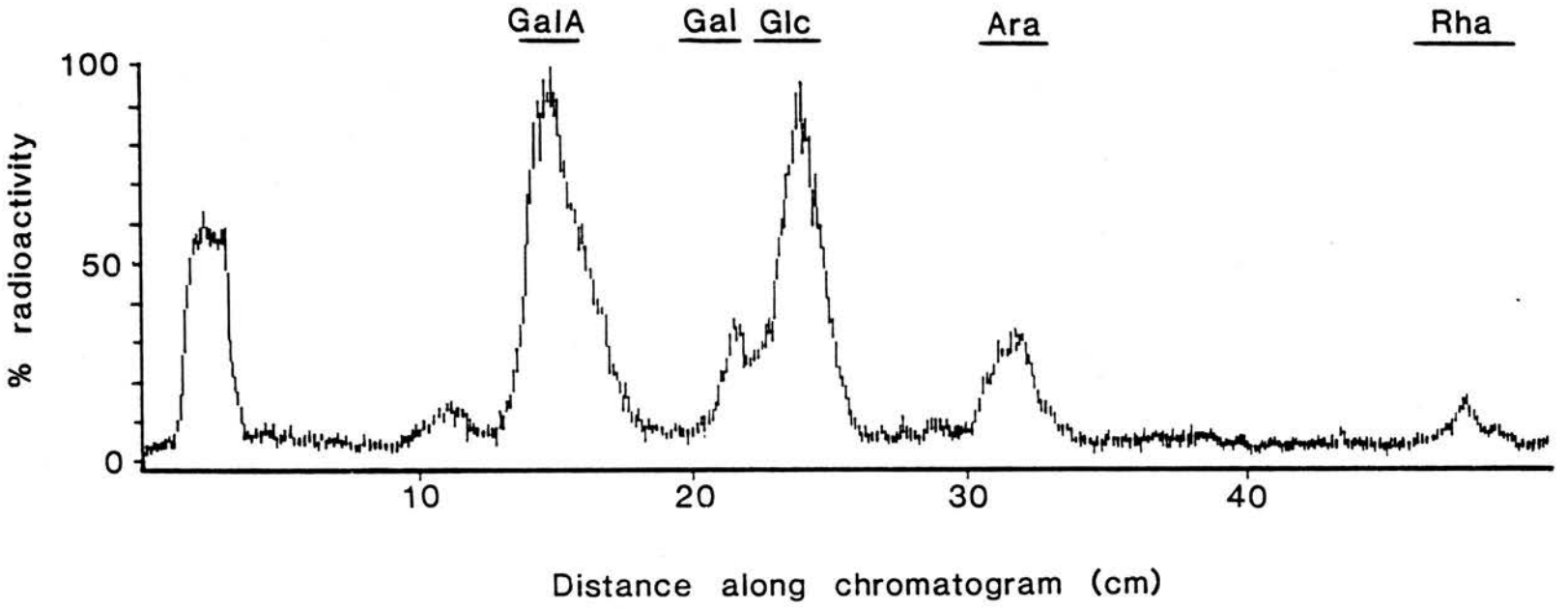


Figure 3.8 : Radioactive profile of the DSF of ^{14}C-plant cell wall preparation I after developmenton paper chromatography in BAW for 16 h and EPW for 16 h.

y-max. (100% radioactivity per channel) = 65 counts

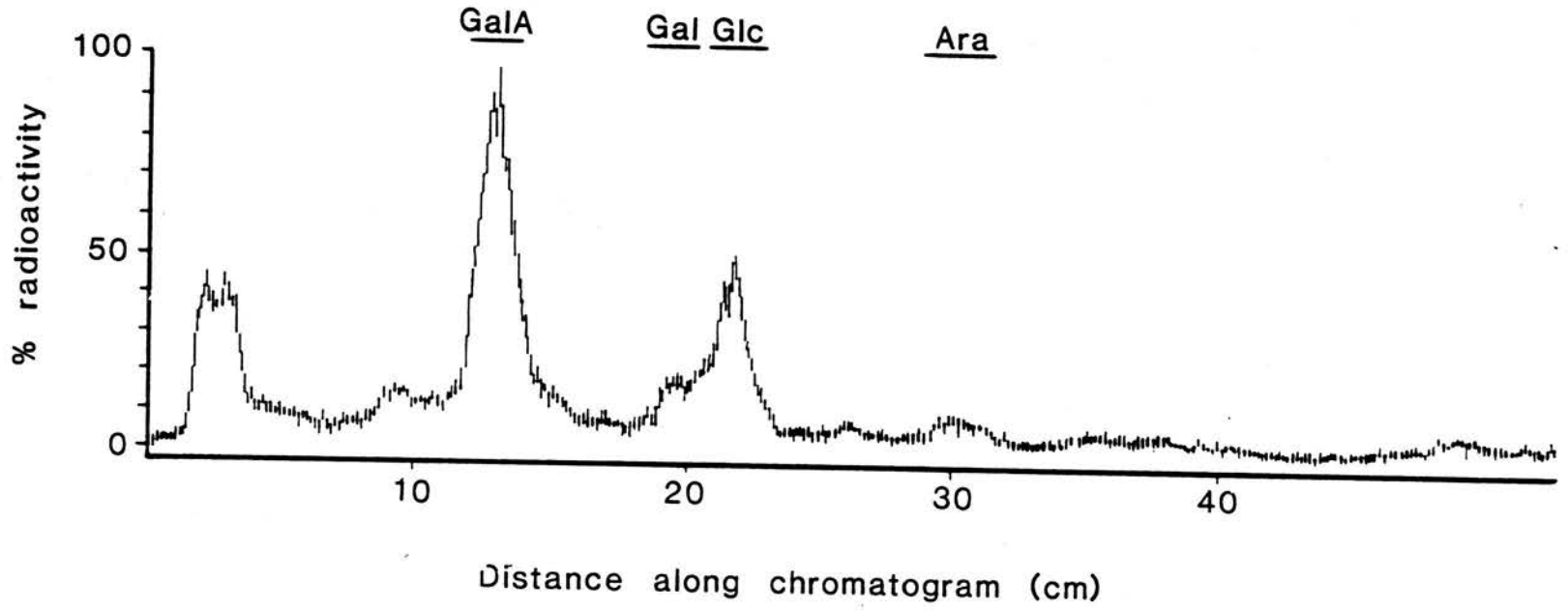


Figure 3.9 : Radioactive profile of the DSF of ^{14}C-plant cell wall preparation II after development on paper chromatography in BAW for 16 h and EPW for 16 h.

y-max. (100% radioactivity per channel) = 103 counts

chromatogram and the XG2 shoulder on the galacturonic acid peak is not visible. Quantification of these peaks was poor using the RITA due to the low and variable counting efficiency (approx. 5%).

A higher counting efficiency was achieved by dividing the chromatogram lanes into 1 or 2 cm strips and determining the activity of each using non-Triton scintillant. From these results, the bar graphs were constructed (Figs. 3.10 and 3.11). The two figures show the presence of the monosaccharides described above and the counts under each peak were determined and expressed as a percentage of the total counts on the chromatogram. Because the total activity of the DSF was determined and the volume loaded onto each chromatogram known, the activity of each [^{14}C]monosaccharide was calculated.

The following results were calculated from at least six different chromatograms and are quoted as the mean value (% of the total ^{14}C on the chromatogram) ± s.e.m. The corresponding activity is given as dpm/mg ^{14}C-plant cell walls.

Table 3.3: ^{14}C-Plant Cell Wall Preparation I

Region on Chromatogram	% ^{14}C	Specific Activity (dpm/mg cell walls)
Origin	4.73 ± 0.04	2.74×10^4
GalA + XG2	43.39 ± 0.14	25.2×10^4
Gal + Glc	31.50 ± 0.15	18.3×10^4
Man	0.57 ± 0.03	0.33×10^4
Ara	9.42 ± 0.05	5.46×10^4
Xyl	1.90 ± 0.04	1.10×10^4
Fuc	1.33 ± 0.05	0.77×10^4
Rha	3.24 ± 0.07	1.88×10^4
Remainder	3.92	2.27×10^4

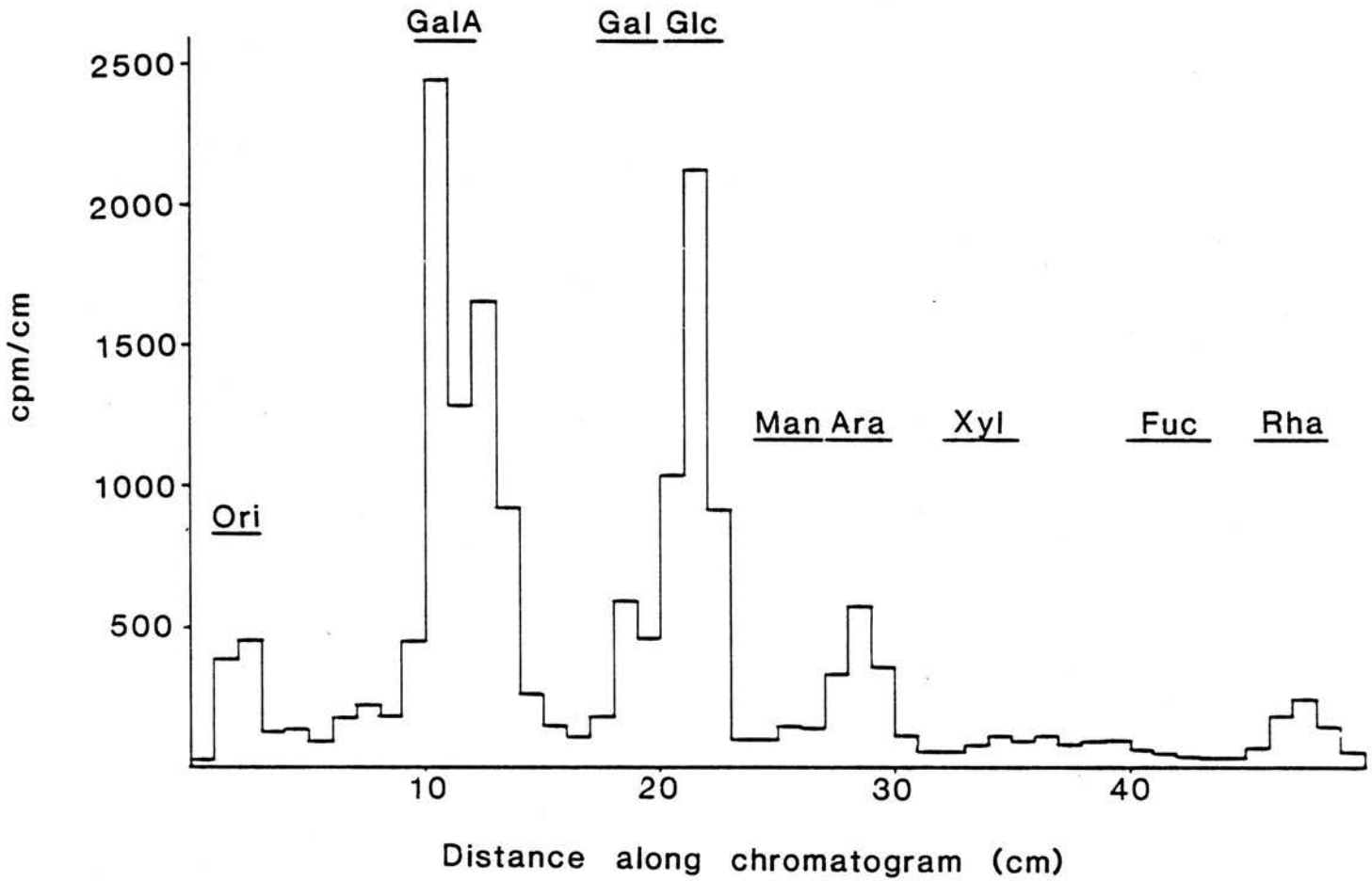


Figure 3.10 : ^{14}C distribution of the DSF of ^{14}C-plant cell wall preparation I after development on paper chromatography in BAW for 16 h and EPW for 16 h.

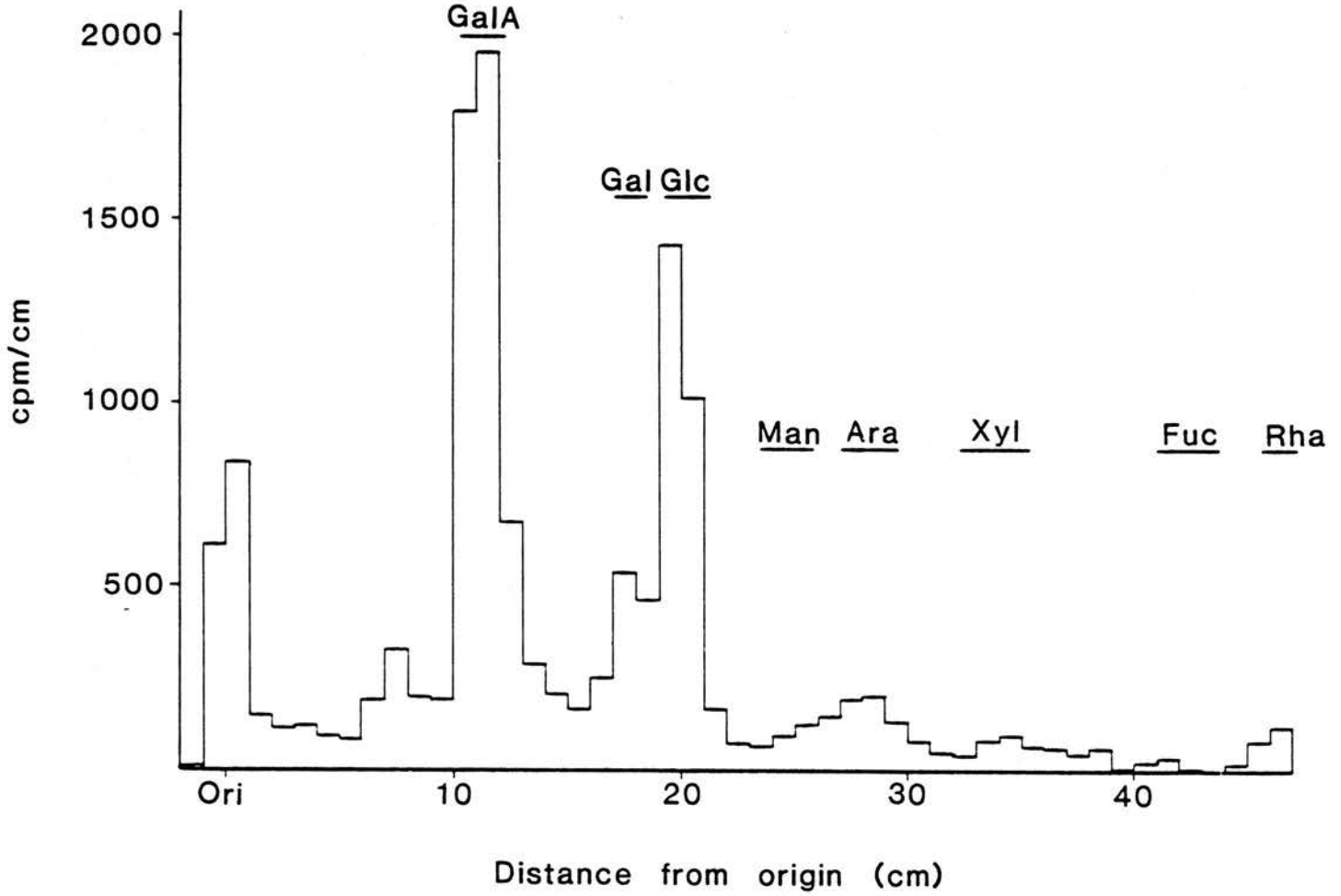


Figure 3.11 : ^{14}C distribution of the DSF of ^{14}C-plant cell wall preparation II after development on paper chromatography in BAW for 16 h and EPW for 16 h.

Table 3.4: ^{14}C-Plant Cell Wall Preparation II

Region on Chromatogram	% ^{14}C	Specific Activity (dpm/mg cell walls)
Origin	9.77 ± 0.06	5.27×10^4
GalA + XG2	38.86 ± 0.20	20.9×10^4
Gal + Glc	28.08 ± 0.13	15.1×10^4
Man	2.48 ± 0.10	1.34×10^4
Ara	5.88 ± 0.09	3.17×10^4
Xyl	3.09 ± 0.06	1.66×10^4
Fuc	1.83 ± 0.09	0.99×10^4
Rha	6.30 ± 0.04	3.40×10^4
Remainder	3.71	2.00×10^4

Both of the ^{14}C-plant cell wall preparations yield two major peaks, which co-chromatograph with the external standards of galacturonic acid and glucose respectively. However the quantification of these peaks is difficult because the disaccharide, XG2, and galactose co-chromatograph so close to the two peaks that it would be inaccurate to separate either XG2 from the galacturonic acid region or galactose from the glucose region. Therefore in the tables above, these regions have been grouped together.

To enable better resolution of these regions in the first ^{14}C-plant cell wall preparation, the galacturonic acid to glucose region was eluted from the chromatogram. The pooled fractions were chromatographed in EPW for 30 h. The lanes of the chromatogram were divided into 1 or 2 cm strips and the activity of each strip was determined by scintillation counting.

Figure 3.12 shows the distribution of the ^{14}C along the

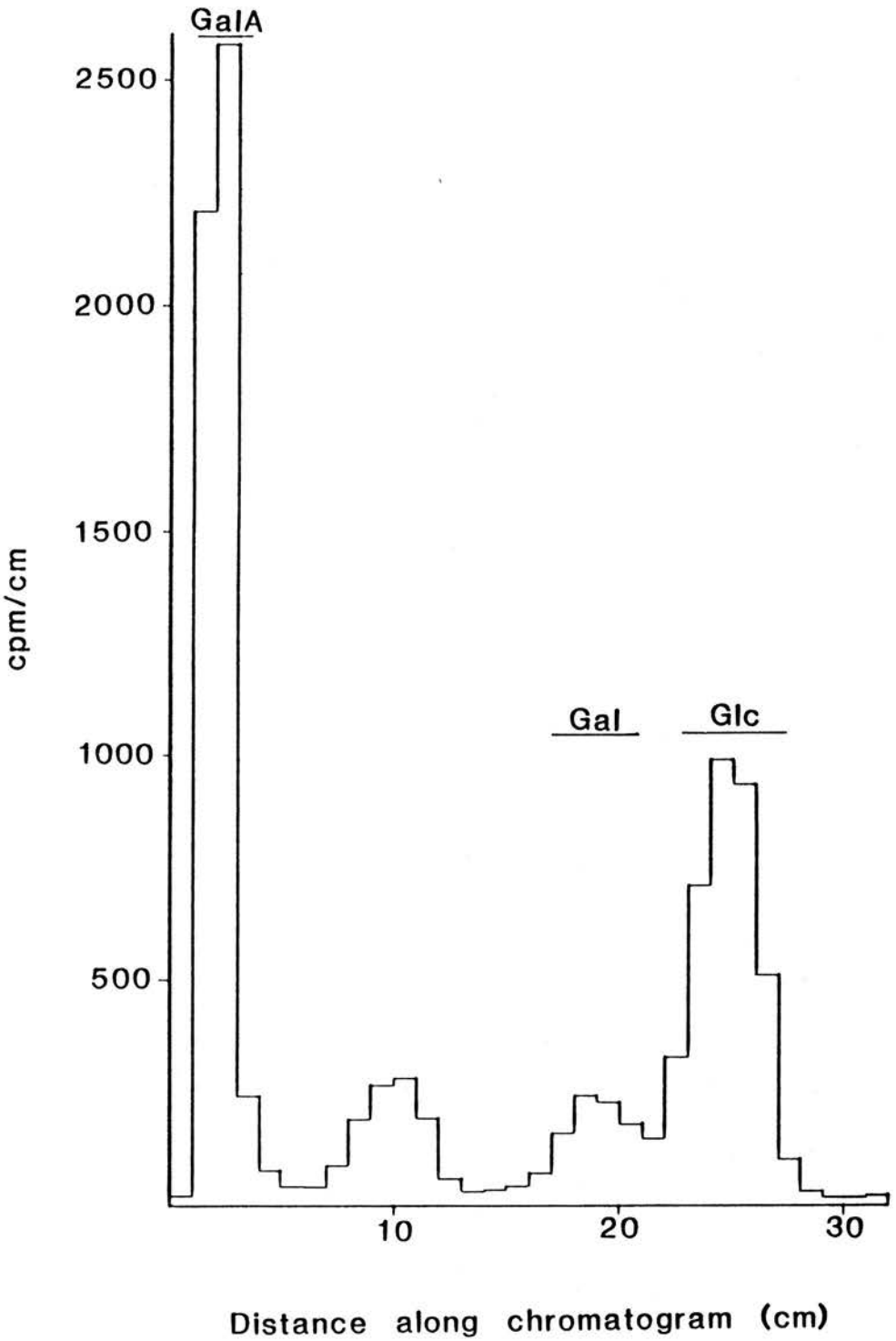


Figure 3.12 : ^{14}C distribution of the GalA to Glc region from Fig. 3.10 after development on paper chromatography in EPW for 30 h.

chromatogram and there are four separate peaks. Three of these co-chromatograph with the external standards shown and the fourth appears in the region where the XG2 would run according to Fry 1988. The peaks described account for more than 97% of the total counts on the chromatogram. The ^{14}C under each peak was calculated as a percentage of the total ^{14}C on the chromatogram and using the results from Fig. 3.8 the activity of the four regions was calculated. The results were calculated from six chromatograms and the values are quoted as the mean value (% of the total ^{14}C on the chromatogram) ± s.e.m. The corresponding activity is given as dpm/mg ^{14}C-plant cell walls.

Table 3.5: ^{14}C Distribution in the Galacturonic acid/Glucose Region

Region of Chromatogram	% ^{14}C	Specific Activity (dpm/mg cell walls)
GalA	46.46 ± 0.41	20.2×10^4
XG2	9.86 ± 0.13	4.29×10^4
Gal	7.48 ± 0.18	3.25×10^4
Glc	33.39 ± 0.40	14.5×10^4
Remainder	2.45	1.07×10^4

The results emphasise that [^{14}C]galacturonic acid and [^{14}C]glucose are the major monosaccharides released after Driselase hydrolysis. This suggests that the ^{14}C-plant cell walls contain mostly pectin, the major source of galacturonic acid, but also significant levels of cellulose.

Acid Hydrolysed Fraction (AHF)

The final stage of the analysis of the ^{14}C-plant cell walls involved the drying under vacuum of the small residue left after Driselase hydrolysis. The residue was HCl hydrolysed and the supernatant analysed. To determine the activity of this fraction, a sample of the supernatant was removed and the volume made up to 1 ml with distilled water. Triton scintillant (10 ml) was added before the activity was determined by scintillation counting. The following results were calculated from at least six measurements and the values quoted are the mean activity (dpm/mg ^{14}C-plant cell walls) ± s.e.m.

Table 3.6: Specific Activities of the AHF

Sample	Specific Activity (dpm/mg cell walls)
^{14}C-plant cell wall prep. I	$1.15 \pm 0.10 \times 10^4$
^{14}C-plant cell wall prep. II	$2.21 \pm 0.06 \times 10^4$

The AHF will contain mostly undigested protein, although there may be some undegraded polysaccharide material as well. The residue remaining after acid hydrolysis was found to have negligible activity. The three fractions analysed (ELF, DSF and AHF) contributed to the total ^{14}C of the ^{14}C-plant cell walls and a summary of the disposition of the ^{14}C in the two individual preparations of ^{14}C-plant cell walls is described in Tables 3.10, 3.11 and 3.12.

Incubation of ^{14}C-Plant Cell Wall Preparation I With Pancreatin

Pancreatin is a commercial mix of enzymes isolated from the pancreas of pigs and normally these enzymes are secreted into the duodenum. Dietary fibre, by definition, is the material which is not digested by the endogenous secretions of the G.I. tract and should therefore remain intact after incubation with this enzyme preparation. The buffer used in this incubation was 0.05 M NaH_2PO_4 containing 0.05% (w/v) sodium azide and the pH adjusted to 7.5 with 0.1 M NaOH. To this was added the Pancreatin at a final concentration of 0.1% (w/v) immediately before use. An accurately weighed amount (approx. 1 mg) of the first ^{14}C-plant cell wall preparation was placed in a Pyrex test tube and 1 ml of the enzyme solution added. The tube was sealed with a Teflon lined cap and incubated at 37°C in a rotary incubator for 16 h. A control tube was set up which omitted the enzyme from the incubation buffer. After the incubation, the suspension was centrifuged to separate the solubilised material from the pellet. Samples of the supernatant were removed and their volume made up to 1 ml with distilled water. Triton scintillant (10 ml) was added before the activity of each sample was determined by scintillation counting.

The results below were calculated from at least four measurements and the figures quoted are the mean values (dpm/mg ^{14}C-plant cell walls). The percentage ^{14}C released by this treatment from the intact cell walls was calculated using the value found for the activity of ^{14}C-plant cell wall preparation I (i.e. 6.45×10^5 dpm/mg cell wall).

Table 3.7: Pancreatin Released ^{14}C from Plant Cell Walls

Sample	Specific Activity of the Supernatant (dpm/mg cell wall)	% ^{14}C Released from the Cell Wall
A	2.09×10^5	32.4
B	2.54×10^5	39.4
Control	2.29×10^5	35.5

These figures suggest that a significant portion of the ^{14}C-plant cell walls was solubilised during the incubation period, although there is little difference between the control and two enzyme samples. Further work was carried out to determine whether the cell wall material was digested to monomer or remained polymeric.

The supernatant was loaded onto a chromatogram and developed in the two solvent system (BAW for 16 h followed by EPW for 16 h) consecutively. Each lane of the paper chromatogram was divided into 1 or 2 cm strips and the activity of each strip determined by scintillation counting (Fig. 3.13)

The radioactive profile of the paper chromatograms was similar in both the control and test samples and only the test sample is shown. The major ^{14}C peak remains stationary at the origin suggesting that there is virtually no breakdown of the ^{14}C-plant cell walls to mono- or disaccharides. A percentage of the ^{14}C-plant cell walls must become solubilised by the incubation conditions but there is no degradation by the Pancreatin.

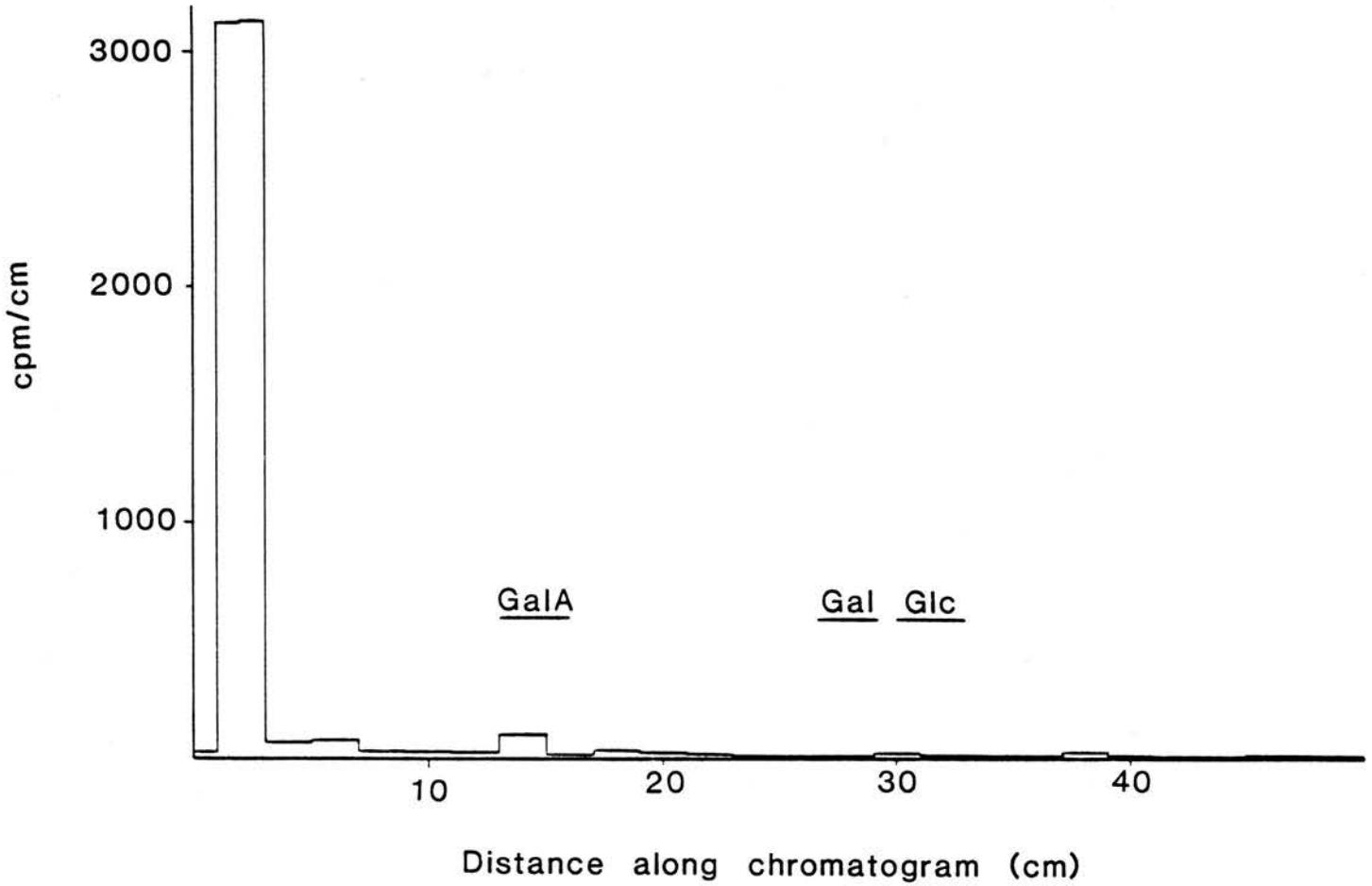


Figure 3.13 : ^{14}C distribution of the pancreatin digested ^{14}C-plant cell wall preparation I after development on paper chromatography in BAW fof 16 h and EPW for 16 h.

[^{14}C]Starch Content in ^{14}C-Plant Cell Wall Preparation II

The presence of significant quantities of [^{14}C]starch would interfere in subsequent feeding studies as some starch is degraded in the upper G.I. tract (stomach and small intestines). This digestion would give a false impression of fermentation in the caecum/colon and hence it was important to determine the level of [^{14}C]starch. A sample of ^{14}C-plant cell walls (approx. 10 mg) was accurately weighed out in a Pyrex test tube. The buffer used for this incubation was 0.2 M sodium acetate (pH 4.5 approx.) and 4.5ml was added to the test tube. The samples were sealed with Teflon lined caps and gelatinised by heating at 100°C for 4 h with regular mixing. After cooling the tube below 50°C, 1 ml of amyloglucosidase (BDH Ltd.) was added and the suspension was incubated at 50°C in a shaking water bath for 16 h. A control tube was set up which contained the buffer and ^{14}C-plant cell walls but no amyloglucosidase. The supernatant was separated from the particulate material by centrifugation at 4,000 rpm. A sample of the supernatant was removed and the volume made up to 1 ml with distilled water. Triton scintillant (10 ml) was added before the activity of the sample was determined by scintillation counting.

The results below were calculated from at least four measurements and the figures quoted are the mean values (dpm/mg ^{14}C-plant cell walls). The percentage ^{14}C released from the intact cell wall was calculated using the value found earlier for the activity of the second ^{14}C-plant cell wall preparation (i.e 5.92×10^5 dpm/mg cell wall).

Table 3.8: Amyloglucosidase Released ^{14}C from Plant Cell Walls

Sample	Specific Activity of the Supernatant (dpm/mg cell walls)	% ^{14}C Released from the Cell Wall
A	1.65×10^5	27.87
B	2.00×10^5	33.78
Control	1.68×10^5	28.38

From these results it was clear that a significant amount of ^{14}C was released from the ^{14}C-plant cell walls during this treatment although, as in the Pancreatin incubation, there is a similar proportion of ^{14}C released from the control (no enzyme) as from the enzyme samples. Further work was carried out to investigate the nature of the solubilised material.

Various amounts of the supernatant were loaded onto a paper chromatogram and run in the EPW solvent system for 16 h. The lanes were divided into 1 cm strips and and the activity of each determined by scintillation counting (Fig. 3.14).

The radioactive profiles of the chromatograms show that the major peak of ^{14}C remains at the origin in all three cases. However there is a small peak of ^{14}C which co-chromatographs with the glucose external marker in the two enzyme samples which is not present in the control sample. The ^{14}C under the glucose peaks was calculated and expressed as a percentage of the total ^{14}C on the chromatograms. The results below were calculated from at least four measurements and are expressed as the mean values (% ^{14}C) ± s.e.m. The percentage ^{14}C occurring as [^{14}C]starch in the plant cell walls was determined and

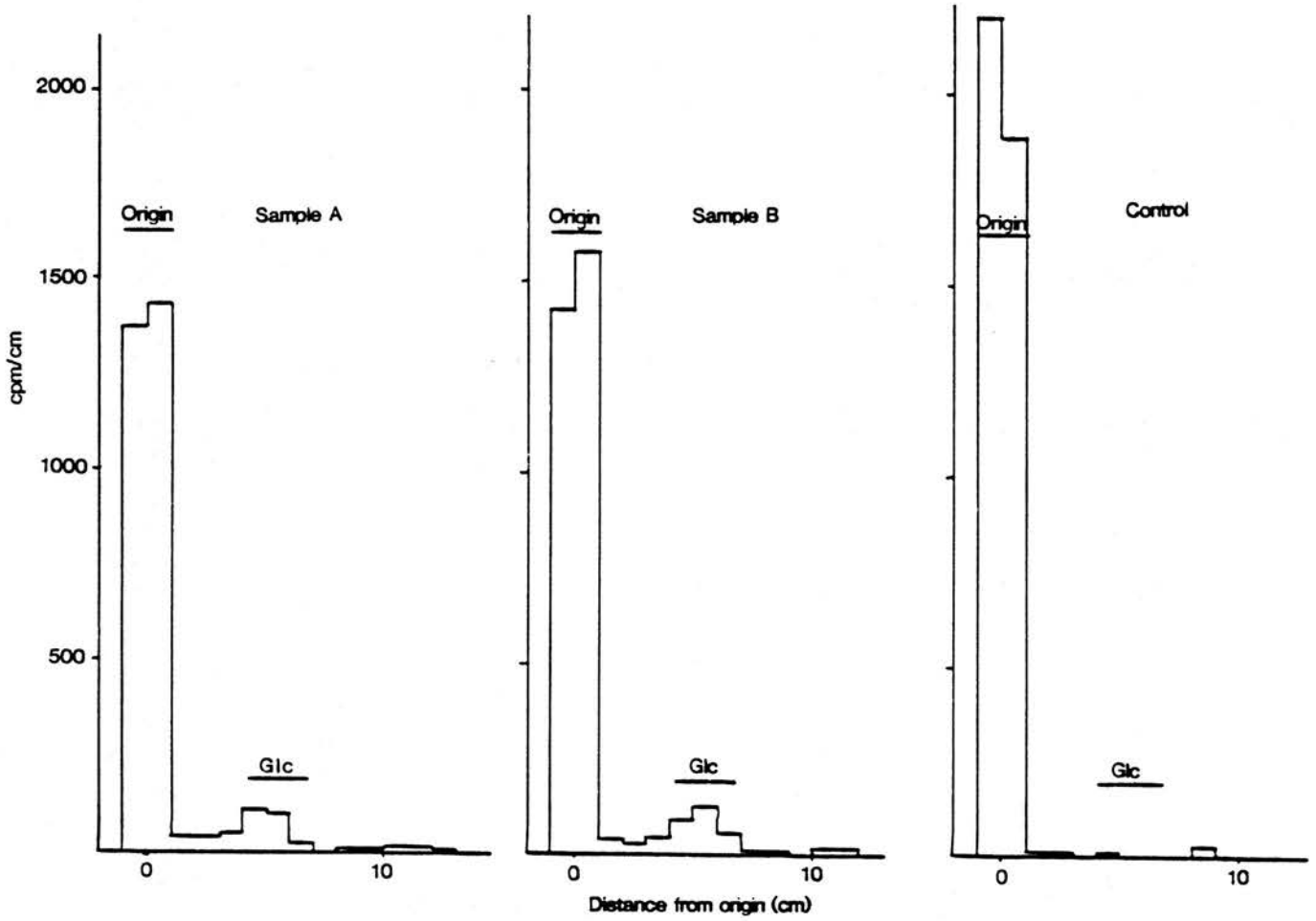


Figure 3.14 : ^{14}C distribution of the amyloglucosidase digested ^{14}C-plant cell wall preparation II after development on paper chromatography in EPW for 16 h.

the corresponding activity calculated as dpm/mg ^{14}C-plant cell walls.

Table 3.9: [^{14}C]Starch in ^{14}C-Plant Cell Walls

Sample	% ^{14}C Released as [^{14}C]glucose	% [^{14}C]starch Released from the Cell Wall	Specific Activity of this [^{14}C]starch (dpm/mg cell wall)
A	8.60 ± 0.24	2.40	1.42×10^4
B	8.40 ± 0.27	2.84	1.68×10^4

These results show that only a small amount on the ^{14}C in the plant cell walls is occurring as [^{14}C]starch and it was concluded that this would not interfere significantly in future feeding studies.

Summary

The purpose of the following tables was to summarise the results obtained in this chapter. From the results in table 3.10 and a knowledge of polysaccharide structure (Chapter 1) it was possible to determine the ^{14}C distribution in the various plant cell wall polysaccharides (Table 3.11). The galacturonic acid residues are associated with the pectic fraction of the cell wall, the majority of which would be homogalacturonan in origin although there would be some associated with rhamnogalacturonan (RG). It has been assumed that all of the rhamnose found came from RG and molar ratios of the RG backbone indicate a 1:1 ratio between rhamnose and galacturonic acid. Virtually all of the methyl ester occurs on the galacturonic acid residues of pectin and this would result in the galacturonic acid residues being 25% methyl esterified. The O-acetyl groups found were distributed in

Table 3.10: Radioactive distribution in ^{14}C-plant cell wall prep. I

FRACTION	RESIDUE	SPECIFIC ACTIVITY (dpm/mg cell walls)	% ^{14}C	MOLAR RATIO*
ELF	**Total**	**5.31×10^4**	**8.24**	-
	methyl ester	0.84×10^4	1.30	1.3
	O-acetyl	2.60×10^4	4.03	2.0
	Other	1.87×10^4	2.90	-
DSF	**Total**	**58.0×10^4**	**89.98**	-
	Ara	5.46×10^4	8.47	1.7
	Fuc	0.77×10^4	1.19	0.2
	Gal	3.25×10^4	5.04	0.8
	GalA	20.2×10^4	31.33	5.2
	Glc	14.5×10^4	22.49	3.7
	Man	0.33×10^4	0.51	0.1
	Rha	1.88×10^4	2.91	0.5
	Xyl	1.10×10^4	1.71	0.3
	XG2	4.29×10^4	6.66	0.6
	Other†	6.20×10^4	9.61	-
AHF	**Total**	**1.15×10^4**	**1.78**	-

* Molar Ratio = $\frac{\%\ ^{14}C \text{ per residue fraction}}{\text{no. of C atoms per residue}}$

† Will include [^{14}C]starch which is poorly digested by Driselase.

TOTAL ACTIVITY : 64.5×10^4 dpm/mg ^{14}C-plant cell walls

Table 3.11: Predicted ^{14}C-distribution in polysaccharide groups [+]

GROUP	RESIDUE	MOLAR RATIO	% ^{14}C
Pectins	Ara	1.5	7.5
	Gal	0.7	4.4
	GalA	5.2	31.3
	Rha	0.5	2.9
	Methyl ester	1.3	1.3
	O-acetyl*	0.5	1.0
	Total		**48.4**
Hemicelluloses	Ara	0.2	1.0
	Fuc	0.1	0.6
	Gal	0.1	0.6
	Glc	0.2	1.2
	Man	0.1	0.5
	XG2	0.6	6.7
	Xyl	0.3	1.7
	O-acetyl*	1.5	3.0
	Total		**15.3**
Cellulose	**Total**	**3.5**	**21.3**
Starch	**Total**		**2.6**

[+] See text for an explanation of the distribution of ^{14}C in the different polysaccharide groups.

* O-acetyl groups are located in both the pectic fraction and the hemicellulosic fraction, although the degree of acetylation varies.

both the pectic and hemicellulosic fraction although extent of this distribution may not be exactly as calculated. In the pectic fraction the O-acetyl groups are attached to the galacturonic acid residues in the RG backbone and the degree of acetylation/methylation of these residues may be variable

The hemicellulosic fraction will have O-acetyl groups attached to the galactose residues of xyloglucan (XG). From the repeating structure of XG (Chapter 1: Fig. 1.2) the ratio of XG2: glucose: galctose: fucose is 6: 2: 1: 1 and hence it is possible to determine the ^{14}C associated with this polysaccharide. The level of XG2 found quantifies the level of the other residues (galactose, glucose and fucose) in the polysaccharide. The xylose produced upon Driselase hydrolysis may have come from arabinoxylan and this accounts for almost 3% of the total ^{14}C in the cell wall.

The two remaining groups in table 3.11 are cellulose and starch. Starch is poorly digested by Driselase (Fry, personal communication) and would not be found as [^{14}C]glucose residues in the DSF. The level of starch was calculated from the results of the amyloglucosidase digestion of ^{14}C-plant cell walls (Fig 3.14). The level of [^{14}C]glucose in the DSF is almost entirely cellulosic in origin although a small amount is released from xyloglucan.

Table 3.12 is a summary of the results obtained from the plant cell wall preparation II. Although the analysis of the ^{14}C-plant cell walls was not as complete as for the first preparation, the overall distribution is similar even if the individual residues vary. Both of the cell wall preparations have similar activities and the predominant residues are galacturonic acid and glucose. The distribution of ^{14}C between the three fractions (ELF, DSF and AHF) is

also similar although the second preparation has a higher proportion in the DSF.

Table 3.12: Radioactive distribution in ^{14}C-plant cell wall prep. II

FRACTION	RESIDUE	SPECIFIC ACTIVITY	% ^{14}C
ELF	**Total**	**3.04×10^4**	**5.14**
	methyl ester	1.46×10^4	2.47
	O-acetyl	0.65×10^4	1.10
	Other	0.93×10^4	1.57
DSF	**Total**	**53.9×10^4**	**91.12**
	Ara	3.17×10^4	5.36
	Fuc	0.99×10^4	1.67
	GalA + XG2	20.9×10^4	35.33
	Gal + Glc	15.1×10^4	25.53
	Man	1.34×10^4	2.27
	Rha	3.40×10^4	5.75
	Xyl	1.66×10^4	2.81
	Other	7.34×10^4	12.41
AHF	**Total**	**2.21×10^4**	**3.74**

TOTAL ACTIVITY : 59.2×10^4 dpm/mg ^{14}C-plant cell walls

There are slight differences between the two preparations (e.g. relative distribution of ^{14}C in the three different fractions) but these differences are quantitative, not qualitative. It was decided that these small differences in the two cell wall preparations were not significant and they were both used in comparable feeding trials. In the following chapter no further distinction is made between the two preparations.

CHAPTER 4 : RESULTS II

FERMENTATION OF [U-^{14}C]SPINACH CELL WALLS IN THE RAT GASTROINTESTINAL TRACT

Aims

The following investigations were conducted to determine the fate of U-^{14}C-labelled spinach cell walls in the rat gastrointestinal tract and to examine the fate of the breakdown products in the expired gases and body tissues.

Introduction

As described in the introductory chapter, comparatively few studies involving the fate of dietary fibre in the gastrointestinal tract have utilised radioactive polysaccharides, and there are no studies involving the use of labelled cell walls as a dietary fibre marker. The ^{14}C-plant cell walls used in these studies were analysed in detail in the previous chapter and were found to contain large quantities of both pectin and cellulose. Although these plant cell walls did not containing every component of fibre, they were used to highlight the important events of dietary fibre metabolism in the G.I. tract. By using a radioactive marker of DF, the animal experiments can be conducted without alterations in the host diet. These problems often arise when assessing the physiological effects of a specific fibre source. The use of ^{14}C-plant cell walls was a novel approach to examine the fate of DF in the rat G.I. tract and could be manipulated and utilised in many different ways. The advantages and potential of this system will be discussed in the next chapter of this thesis.

Pilot Study

An initial study was conducted to determine the extent of recovery of the ^{14}C-plant cell walls from rats housed in metabolism cages. The trial period lasted 4 days to maximise the output of ^{14}C

and enable complete fermentation of the cell walls. Three rats were fed by gavage (force fed) with a known activity of the ^{14}C-plant cell walls and housed separately in the metabolism cages. After 4 days the animals were killed and dissected .

The following results (Table 4.1) show the distribution of ^{14}C in the faeces, urine, caecum and selected body tissues of the rats. The values quoted are the average of two measurements from each sample and are a percentage of the dosed activity. The last column in the table shows the average for the three rats ± the standard error of the mean (s.e.m.).

Table 4.1: Distribution of ^{14}C in Rats After 4 Days

Sample	Rat A (% ^{14}C)	Rat B (% ^{14}C)	Rat C (% ^{14}C)	Average ± s.e.m. (% ^{14}C)
Faeces				
Day 1	10.86	16.43	20.33	15.87 ± 1.59
Day 2	2.51	1.17	1.20	1.63 ± 0.26
Day 3/4	0.47	0.64	0.70	0.60 ± 0.04
Caecal cont.	0.07	0.06	0.07	0.07 ± 0.01
Urine				
Day 1	0.70	3.06	0.81	1.52 ± 0.44
Day 2	0.21	0.31	0.31	0.28 ± 0.02
Day 3/4	0.21	0.17	0.23	0.20 ± 0.01
Tissues				
Liver	1.06	1.25	1.42	1.24 ± 0.06
Caecum	0.14	0.09	0.23	0.15 ± 0.02
Adipose	0.21/g	0.32/g	0.23/g	0.25/g ± 0.02
Skin	0.15/g	0.11/g	0.12/g	0.13/g ± 0.01
Body weight	190 g	162 g	204 g	

The table above shows that the ^{14}C-plant cell walls are extensively broken down in the rat G.I. tract, with only 20% of the ^{14}C excreted in faeces and urine over the 4 day period. The low levels of ^{14}C in the caecal contents suggests that virtually all of the ^{14}C has passed out of the G.I. tract, although there might be some in the

colon. There were particles in the urine samples, suggesting contamination from the faeces and for this reason, future work combined the urine and faecal activities. The incorporation of ^{14}C into the various host tissues is confirmation that the ^{14}C-plant cell walls were broken down in the G.I. tract and that the break down products were subsequently utilised by the host.

The figures quoted in table 4.1 account for only 22% of the total ^{14}C dose although there might be some activity in other tissues which were not examined. By using the total body weight of the rats and the adipose tissue values as an indicator of ^{14}C distribution in other tissues not examined, the % recovery of ^{14}C was calculated as 68% of the ^{14}C dose. This would indicate that a significant portion of the ^{14}C can be attributed to the remaining carcass although the exact amount might be considerably different from the level calculated. Of the 20% ^{14}C excreted in the 4 day trial period the majority (>80%) was recovered in the first 24 h.

24 Hour Time-Course Study

From the results of the pilot study, it was clear that the first 24 h after gavage were the most important and so this period was investigated in more detail. Three rats were assigned to each of the sample times (3, 6, 12 and 24 h) and received measured doses of the ^{14}C-plant cell walls. After dosing, the animals were placed into individual metabolism cages for the duration of the experiment. The animals were killed at the appropriate sample time and dissected immediately. Table 4.2 show the ^{14}C distribution in the G.I. contents, faeces, various tissues and the remaining carcass for the 4 time groups. The values quoted are the averages of the three rats in each

group and are expressed as a percentage of the total ^{14}C dose.

Table 4.2: Distribution of ^{14}C in Rats During the Time-Course Study

SAMPLES	SAMPLE TIMES (HOURS) 3 (% ^{14}C)	6 (% ^{14}C)	12 (% ^{14}C)	24 (% ^{14}C)
Stomach contents	16.37	14.96	0.18	ND*
Small Intestine contents	95.23	11.28	2.36	1.44
Caecal Contents	3.92	17.77	13.10	2.00
Colon Contents	2.73	3.46	10.41	1.39
Faeces	0.64	10.19	4.83	17.56
Tissues				
Stomach	0.22	0.25	0.18	ND
Small Intestines	0.63	0.87	0.84	1.53
Caecum	0.10	0.41	0.25	0.26
Colon	0.10	0.51	0.56	0.33
Liver	2.42	2.25	3.61	1.92
Skin	3.16	2.14	4.87	4.01
Carcass	5.65	5.42	10.13	12.44
Recovery	132.07	69.51	51.32	42.88

* Not done

The values from the table above are also represented in figures 4.1 and 4.2 which show graphically the ^{14}C distribution of each sample over 24 h.

The measurement of freeze-dried plant cell walls was difficult, due to the nature of the material and the small quantities used (<10 mg). The administration of the ^{14}C-plant cell walls to the rats was another source of error and together they contribute to the variation observed in these experiments. The recovery of ^{14}C in the 3 hour group was in excess of 100% and was due to one of the animals receiving an excess of the ^{14}C-plant cell wall material. This did inflate the values obtained for this group but the animal did exhibit a similar pattern to the rest of the group and was therefore included in the study. However it was still possible to draw meaningful conclusions from these results.

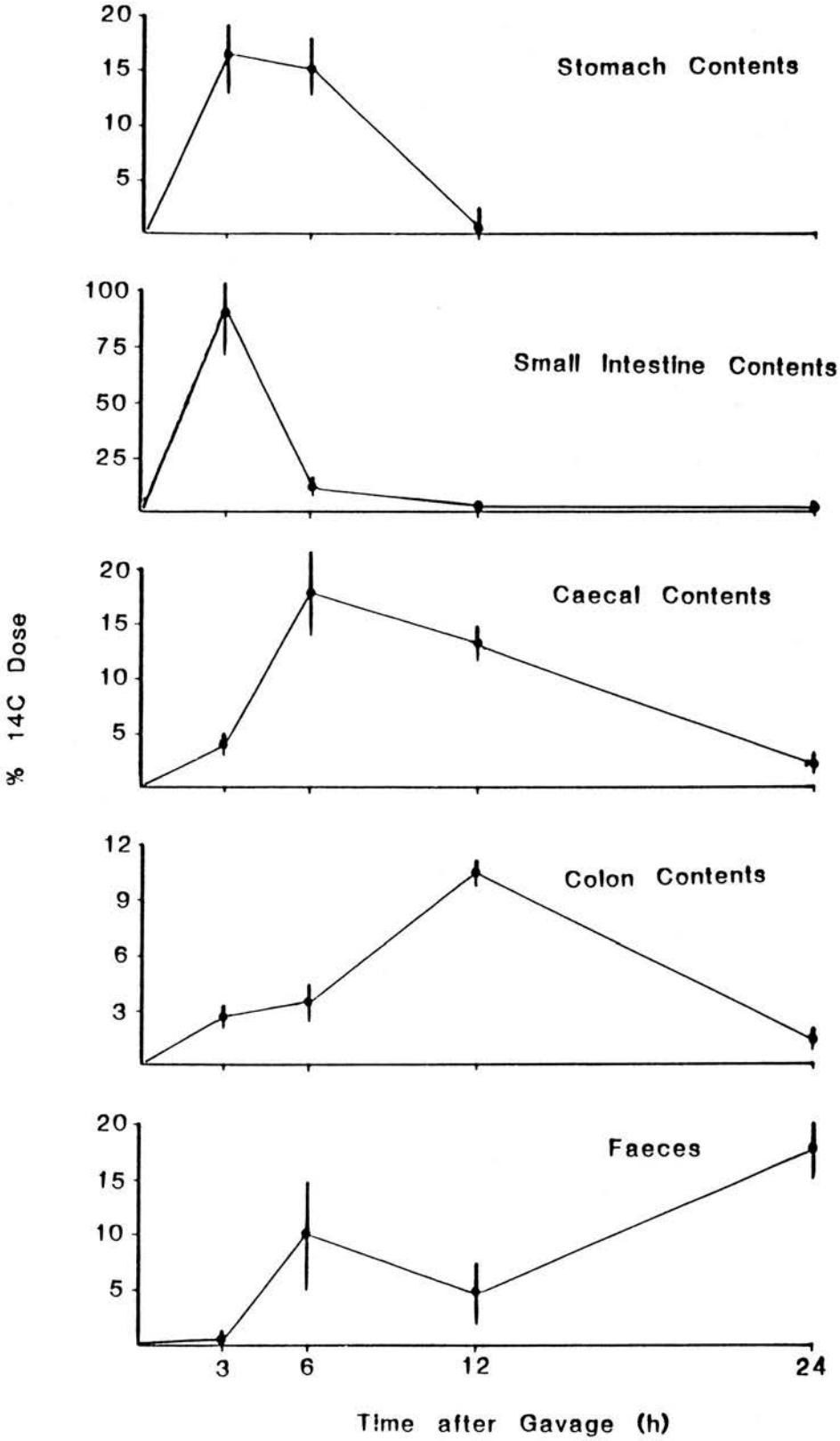
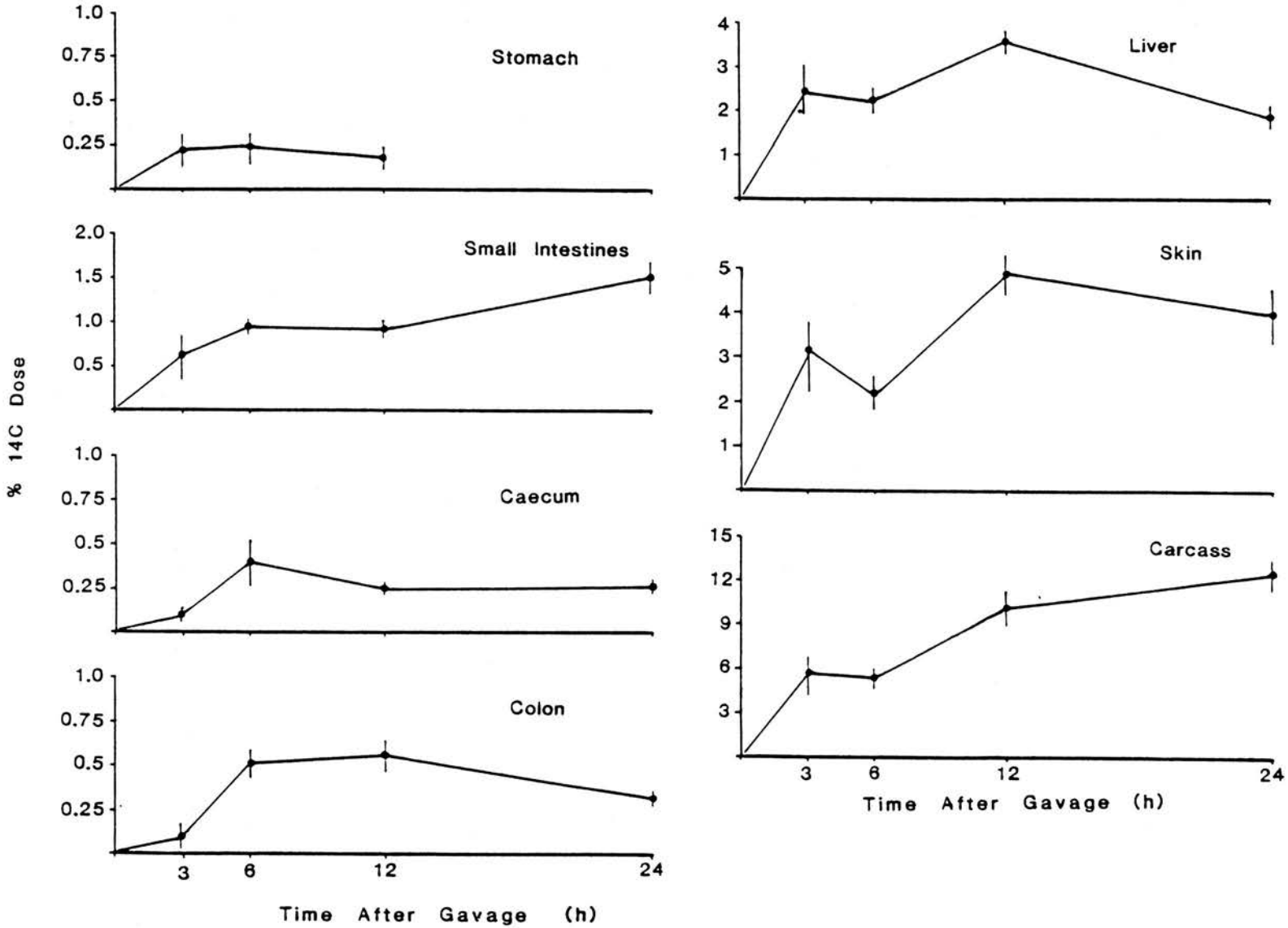


Figure 4.1 : Distribution of ^{14}C-plant cell walls in the G.I. tract of rats over 24 hours after gavage with ^{14}C-plant cell walls.
Bars = s.e.m.

Figure 4.2 : Distribution of ^{14}C in the rat tissues over 24 hours after gavage with ^{14}C-plant cell walls.
Bars = s.e.m.


% 14C Dose
Stomach
Small Intestines
Caecum
Colon
Liver
Skin
Carcass
1.0
0.75
0.5
0.25
2.0
1.5
1.0
0.5
4
3
2
1
5
4
3
2
1
15
12
9
6
3
3
6
12
24
Time After Gavage (h)

After 3 h, most of the ^{14}C was found in the small intestines but the presence of ^{14}C in the liver tissue indicated that there was already some break down of the ^{14}C-plant cell walls. This slight breakdown may have occurred in the small intestines by the degradation of the small amount of starch present or by the fast transit of the liquid phase of the diet, which may carry some of the soluble fibres. There appears to be some activity in the skin after 3 h, but this might be due to blood contamination of the skin at dissection.

By 6 h the ^{14}C was more evenly distributed along the G.I. tract, although one of the animals had a significant portion of the ^{14}C in the faeces (approx. 30% of the ^{14}C dose). In these studies it was unusual to detect significant ^{14}C activity in the faeces after 6 h and this might be due to a rapid transit time. However this unusual result shows that the fast transit time reduces the extent of bacterial fermentation, approx. 30% of the ^{14}C is excreted compared to normal transit time when only 20% of the ^{14}C dose is excreted. There is also an increase of ^{14}C in the caecal and colon tissue at 6 h and in the colon tissue at 12 h. The subsequent decrease in these values after 24 h and might be caused by the direct incorporation of the ^{14}C fermentation products (SCFA) into the mucosal wall and their subsequent metabolism. It has been reported that intestinal tissues can utilise butyrate as an energy source directly from the lumen of the G.I. tract (Sakata 1987).

After 12 h the majority of the ^{14}C has moved into the lower G.I. tract (caecum and colon) and it is in these areas that the bacteria are most abundant and most active. This is confirmed by a peak of ^{14}C in the liver (maximum uptake of ^{14}C-breakdown products) which decreases at 24 h. By 24 h, a significant proportion of the

recovered ^{14}C has been excreted in the faeces. In the skin and carcass there is a accumulation of ^{14}C indicating that there is incorporation of the radioactive label but no subsequent respiration. Over the 24 h period there is a gradual reduction in ^{14}C recovered and it was thought that this loss may be due to production of $^{14}CO_2$. This hypothesis is examined in a later section of this chapter.

These experiments have shown the passage of the ^{14}C-plant cell walls and their subsequent metabolism in the G.I. tract without showing detail of the cell wall structure. An advantage of using intact plant cell walls was the presence, in their natural state, of different plant polysaccharides (pectins, hemicelluloses and cellulose). The following section investigates the fate of the individual plant polysaccharides as the ^{14}C-plant cell walls pass through the G.I. tract.

Degradation of ^{14}C-Plant Cell Wall Polysaccharides

In order to detect the individual plant polysaccharides in the G.I. contents, a 10-fold increase in the activity of the ^{14}C-plant cell walls administered was required. By killing animals at various time intervals, indicated by the 24 h time-course experiments above, the ^{14}C profile in different parts of the G.I. tract was investigated. The G.I. contents of the animals were removed and frozen immediately prior to freeze-drying. For the analysis, the freeze-dried material was treated in the same way as the ^{14}C-plant cell wall preparations outlined in the previous chapter. The first stage was mild alkali hydrolysis and this was followed by Driselase digestion. It was the driselase soluble fraction (DSF) which provided the information concerning the fate of the individual plant polysaccharides. The DSF

of the G.I. contents was loaded onto a paper chromatogram and developed in both BAW and EPW consecutively. The individual lanes of the chromatogram were divided into 1 cm strips and the activity of each was determined by scintillation counting. Monosaccharide standards were run in lanes adjacent to the DSF and used as indicators.

Figure 4.3 shows the radioactive profiles of these chromatograms and the profile obtained from ^{14}C-plant cell wall preparation II. All the peaks are expressed as a percentage of the total ^{14}C on the chromatogram and the x-axis shows R_{Rha} values (mobility relative to that of rhamnose). The use of R_{Rha} values should bring the other peaks into line although there may be some slight discrepancies because external standards do not always precisely reflect the position of the monosaccharides in the test lanes. However, the major peaks are easily identified and conclusions can be drawn from these profiles. The explanations of the profiles will concentrate on the fate of the two major polysaccharide fractions present in the ^{14}C-plant cell walls; the pectic fraction (galacturonic acid) and the cellulose fraction (glucose). The other peaks described in the previous chapter were difficult to detect after passing along the G.I. tract of the rat and future work to investigate their fate would have to include higher doses of ^{14}C-plant cell walls.

In the cell wall profile the galacturonic acid peak was considerably larger than the glucose peak and this was also the case in the stomach and small intestines. The shapes of the peaks are not necessarily identical and were dependent on how the samples ran and where the separate 1 cm strips were cut. The peak of ^{14}C at the origin was probably due to a combination of factors; undegraded glycoprotein,

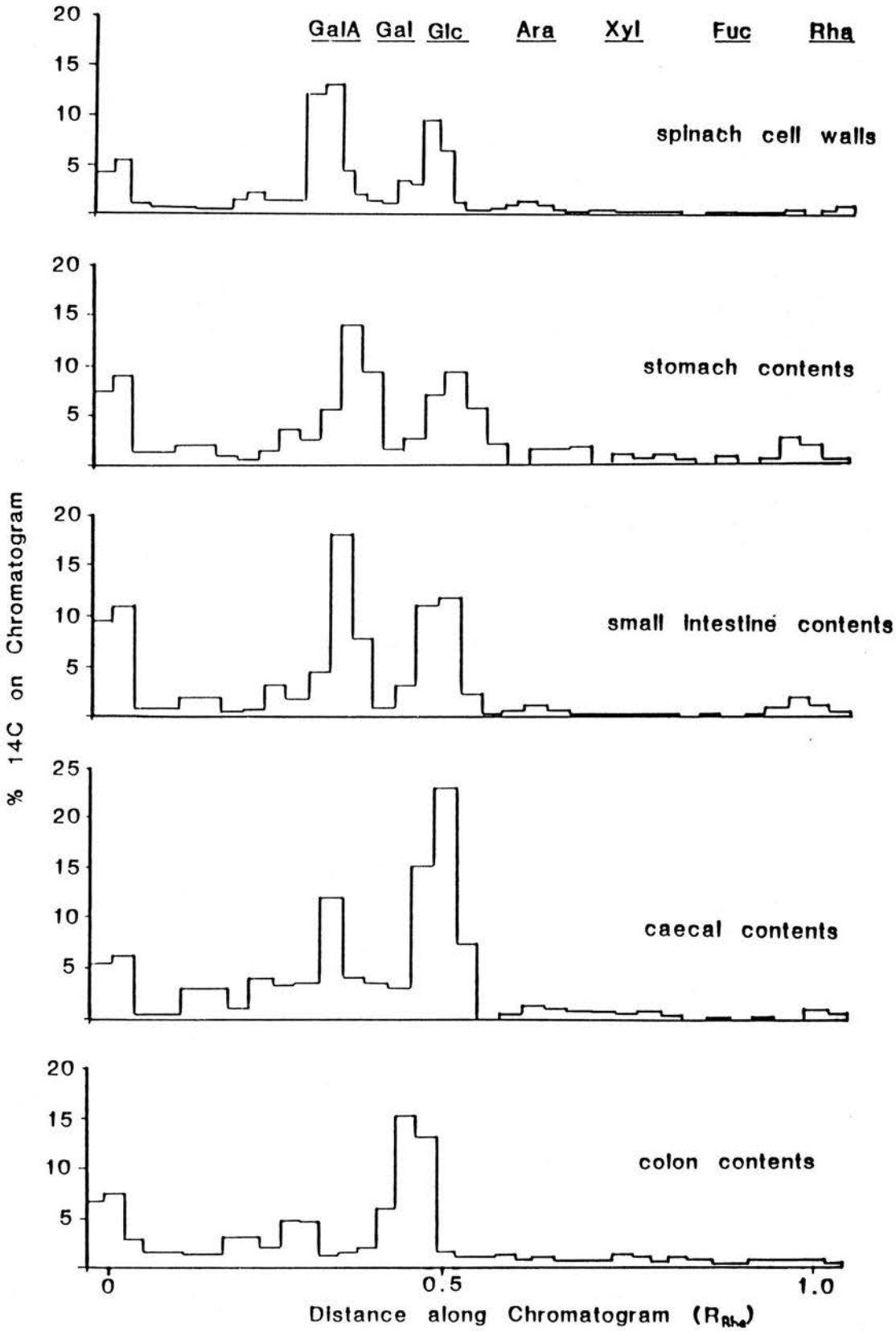


Figure 4.3 : Paper chromatography profiles of Driselase digested contents of the G.I. tract of rats fed ^{14}C-plant cell walls compared to the ^{14}C-plant cell walls prior to ingestion. The difference between chromatograms has been corrected using the mobility of rhamnose (R_{Rha}) as a standard.

protein and perhaps even a small amount of residual polysaccharide. There might also have been insoluble material produced after loading the DSF onto the chromatogram, producing a strong association between the sample and the cellulose paper, and this will depend on the sample size loaded.

The important changes are seen when the caecal contents are examined and these show that the glucose peak was far larger than the galacturonic acid peak. This does not indicate much about the fate of the cellulose fraction, as absolute values were not calculated, but does confirm that the pectic fraction was extensively fermented in the caecum. This fermentation continues in the colon, although most bacterial activity will be confined to the proximal colon, where the pectic fraction was completely removed. After fermentation of the ^{14}C-plant cell wall, some of the ^{14}C might become incorporated into bacterial components. Some of these ^{14}C-labelled bacteria would be flushed out of the G.I. tract in the faecal material and would be indistinguishable from undegraded ^{14}C-plant cell wall components, because only total ^{14}C was measured. In the ^{14}C-plant cell wall there was only 21.3% [^{14}C]cellulose and yet approximately 20% of the ^{14}C dose was recovered in the faeces. This suggests that there was probably some cellulose degradation in the G.I. tract, though the percentage cellulose recovered in the faeces was not determined.

24 Hour $^{14}CO_2$ Production in Rats

As shown in the previous two sections, there was fermentation of the ^{14}C-plant cell walls by the bacteria in the caecum of the G.I. tract and incorporation/metabolism of the radioactive products by the host tissues. These processes will produce energy and

significant amounts of carbon dioxide as an end product. It was postulated that the decrease in the recovery of ^{14}C, during the time-course experiments, was due to the loss of $^{14}CO_2$. This was investigated in the following series of experiments.

To determine the amount of $^{14}CO_2$ produced in rats during the first 24 h, the animals were housed separately in the metabolism/respiration cages (Fig 2.1) after gavage with the ^{14}C-plant cell walls. From each of the two CO_2 traps, 1 ml of Carbosorb (Packard Instruments) was removed every hour and added to 7 ml of Carbosorb in a scintillation vial. Before scintillation counting, 10 ml of Permafluor was added to each vial. Figure 4.4 shows the percentage ^{14}C released in the form of $^{14}CO_2$ over the 24 h period and is the average value of three rats.

The production of $^{14}CO_2$ will depend on the stomach to caecal transit time, but in all the studies that were carried out the pattern of $^{14}CO_2$ production was similar. There was a delay before the start of $^{14}CO_2$ production which would be due to the delay in the transit time of the ^{14}C-plant cell wall material. The production of $^{14}CO_2$ reached its mximum rate 4 h and 9 h. Although the rate of $^{14}CO_2$ production fell significanctly after 13 h, there was still a gradual increase in the total $^{14}CO_2$ produced for the entire 24 h. The increase in $^{14}CO_2$ produced from 13 to 24 h reflects the slow turnover/metabolism of the ^{14}C incorporated into the host tissues observed during the time-course study. From these studies it was not possible to determine the source of the $^{14}CO_2$ (i.e. bacterial or host activity).

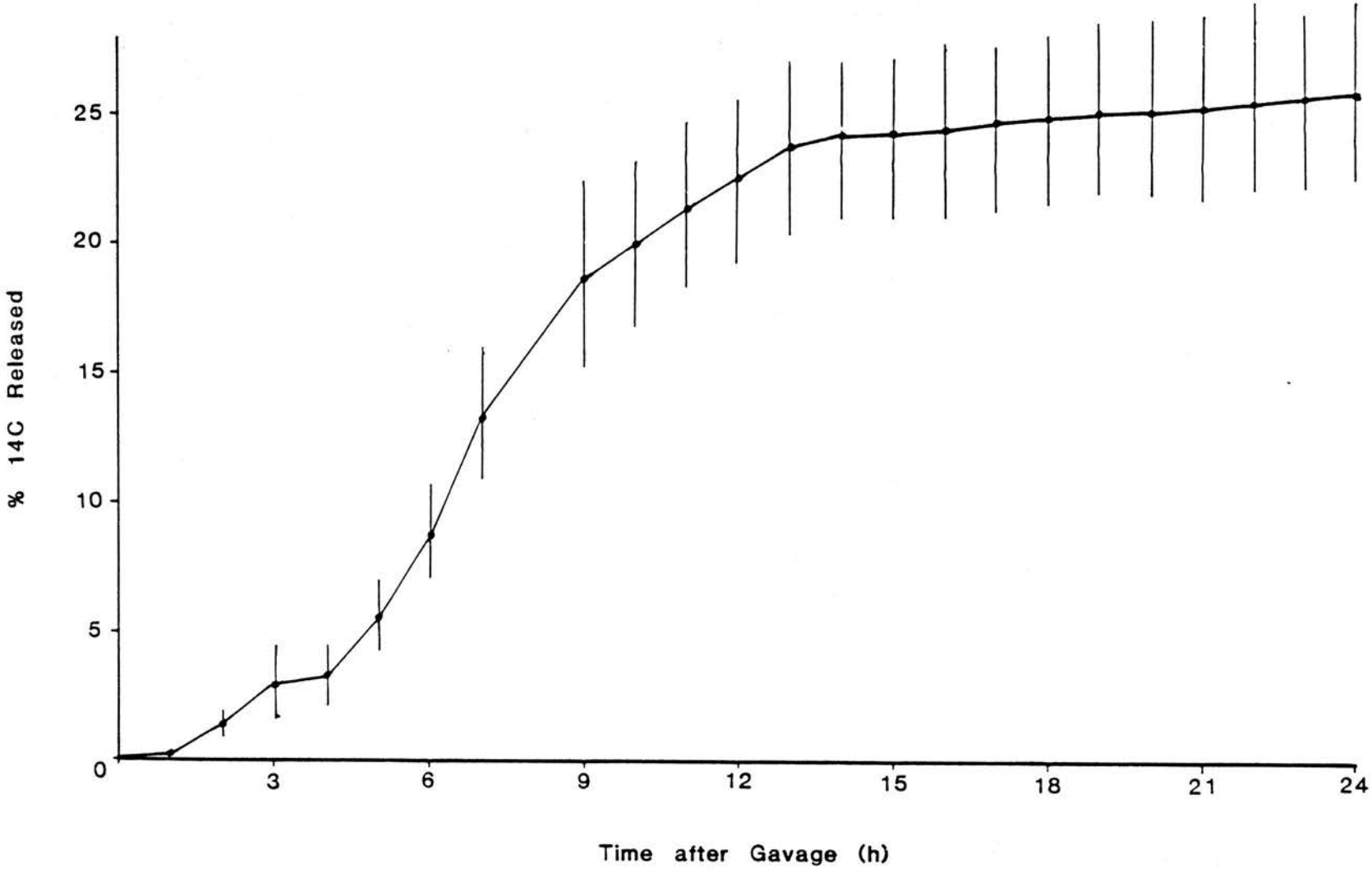


Figure 4.4 : Production of $^{14}CO_2$ from rats fed a high fibre diet after gavage with ^{14}C-plant cell walls.
Bars = s.e.m.

Bacterial Fermentation *in Vitro*

The caecal bacterial activity was investigated separately from the animal model using a minimal salts medium to reduce the fermentable substrate available. If a complex medium was used (e.g. reinforced clostridial medium) the fermentation of the plant cell walls may be obscured by the fermentation of other components of the medium. It was felt that in the time available, more useful information could be derived using the medium and system outlined. It is accepted that many different *in vitro* approaches could be applied to the problem of investigating the complex fermentation of ^{14}C-plant cell walls. The test flasks contained the caecal inoculum and 1% plant cell walls whereas the control flasks contained only the inoculum. There was some residual fibre in the caecal inoculum which would be available for fermentation as well as a significant level of SCFA. However the SCFA level in the inoculum or in the flasks immediately after inoculation was not determined. The reason for this was to maintain an anaerobic conditions in the flasks and allow the bacteria time to adapt to the change in environment.

Figure 4.5 shows the production of $^{14}CO_2$ in the fermentation flasks and was obtained from two identical flasks. Values are quoted as a percentage of the total ^{14}C dose. From this graph it was apparent that the bacteria adapt quickly to their new environment and begin to ferment the ^{14}C-plant cell walls immediately. There was a linear rate of production of $^{14}CO_2$ at the beginning, with the maximum rate between 3 and 9 h after inoculation. Between 9 and 24 h the production rate had fallen, although there was still some $^{14}CO_2$ produced. The level of $^{14}CO_2$ produced *in vitro* (approx. 15% of the ^{14}C dose) was considerably less than the level produced *in vivo* (approx. 25% of the ^{14}C dose),

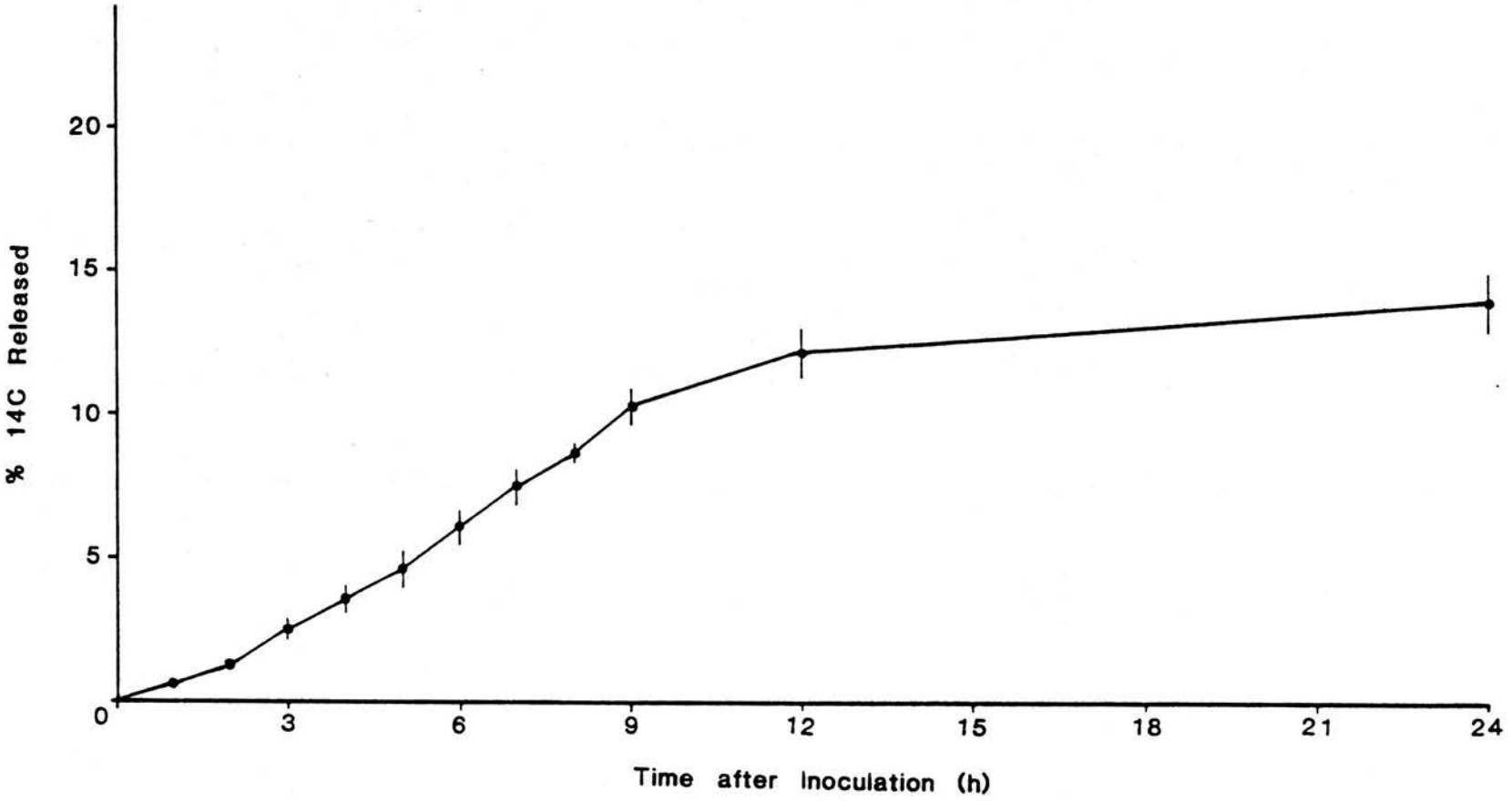


Figure 4.5 : Production of $^{14}CO_2$ from *in vitro* fermentations containing ^{14}C-plant cell walls and a high fibre inoculum over 24 hours. Bars = s.e.m.

and so direct comparisons should be treated with caution. The differences in the two systems are explained in more detail in the discussion chapter. In these flasks, the level of SCFA was determined also.

Due to the methodology available it was not possible to measure the level of [^{14}C]SCFA but it was assumed that the ^{14}C-plant cell walls would yield the same SCFA profile as the non-radioactive cell wall material. Using this assumption any future discussion concerning the level of non-radioactive SCFA will be directly comparable to the level of [^{14}C]SCFA produced from the fermentation of the ^{14}C-plant cell walls. At 3, 6, 9, 12 and 24 h, 1 ml of the culture supernatant was removed and the quantity of SCFA determined. Figures 4.6 - 4.10 show the total SCFA and the four major SCFA (acetate, propionate, butyrate and valerate) produced during 24 h.

From the graphs it was apparent that there was a difference in the level of SCFA produced between the control flask and the test flasks with the exception of the valerate production (Fig. 4.10). In the graph showing total SCFA produced (Fig. 4.6) there was a two-fold increase in the flasks containing 1% plant cell wall material compared to the control flask. It was thought that this increase resulted from the fermentation of the plant cell wall material. By far the most abundant SCFA, in this system, was acetate which accounted for more than half of the total SCFA produced. In descending order of abundance were acetate, propionate, butyrate and valerate. As with the $^{14}CO_2$ production, the SCFA production was more pronounced in the first 12 h than in the second 12 h, although it would have been an advantage to determine the SCFA level at inoculation to establish the t = 0 h points. These results indicate that the bacteria can ferment ^{14}C-plant

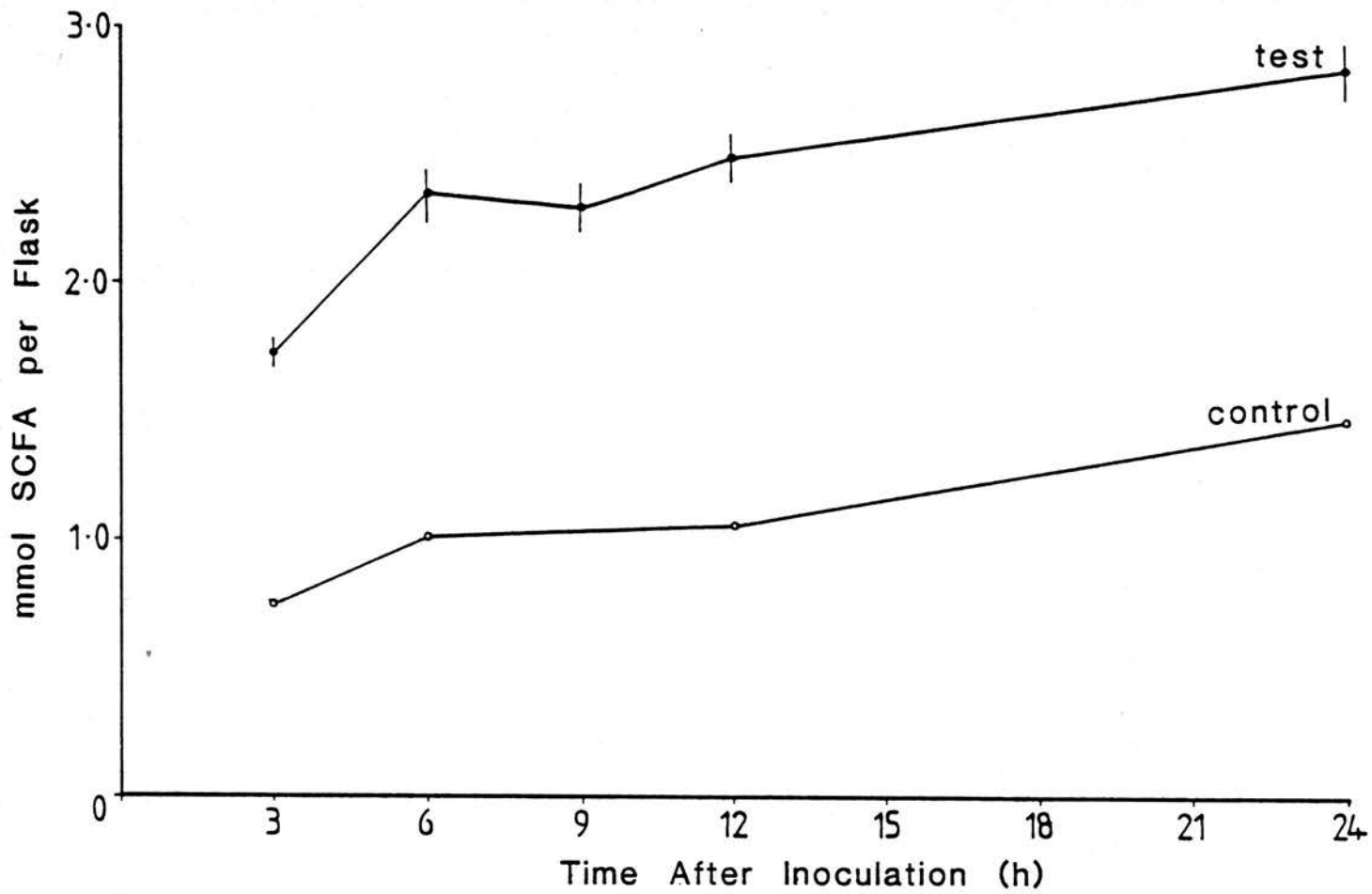


Figure 4.6 : Total production of SCFA from *in vitro* fermentations containing ^{14}C-plant cell walls and a high fibre inoculum over 24 hours.

Bars = s. e. m.

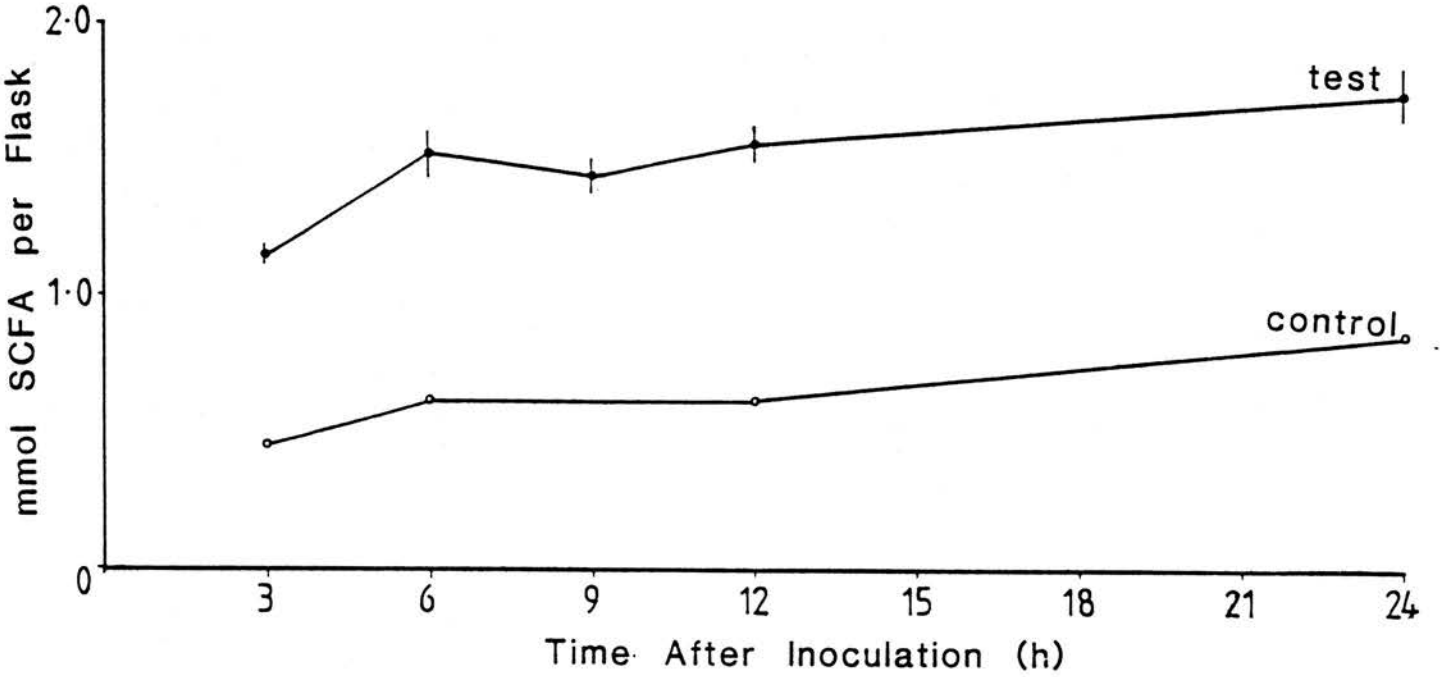


Figure 4.7 : Production of acetate from *in vitro* fermentations containing ^{14}C-plant cell walls and a high fibre inoculum over 24 hours.

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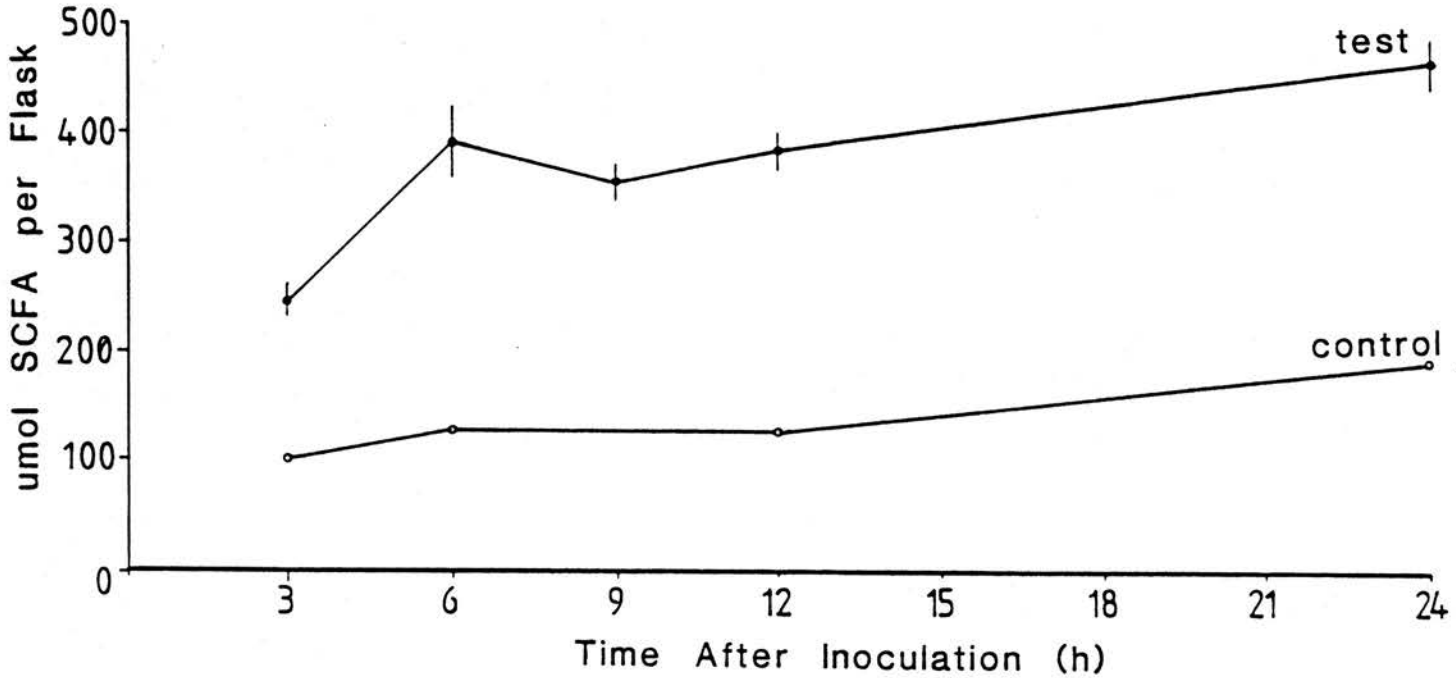

Figure 4.8 : Production of propionate from *in vitro* fermentations containing ^{14}C-plant cell walls and a high fibre inoculum over 24 hours.

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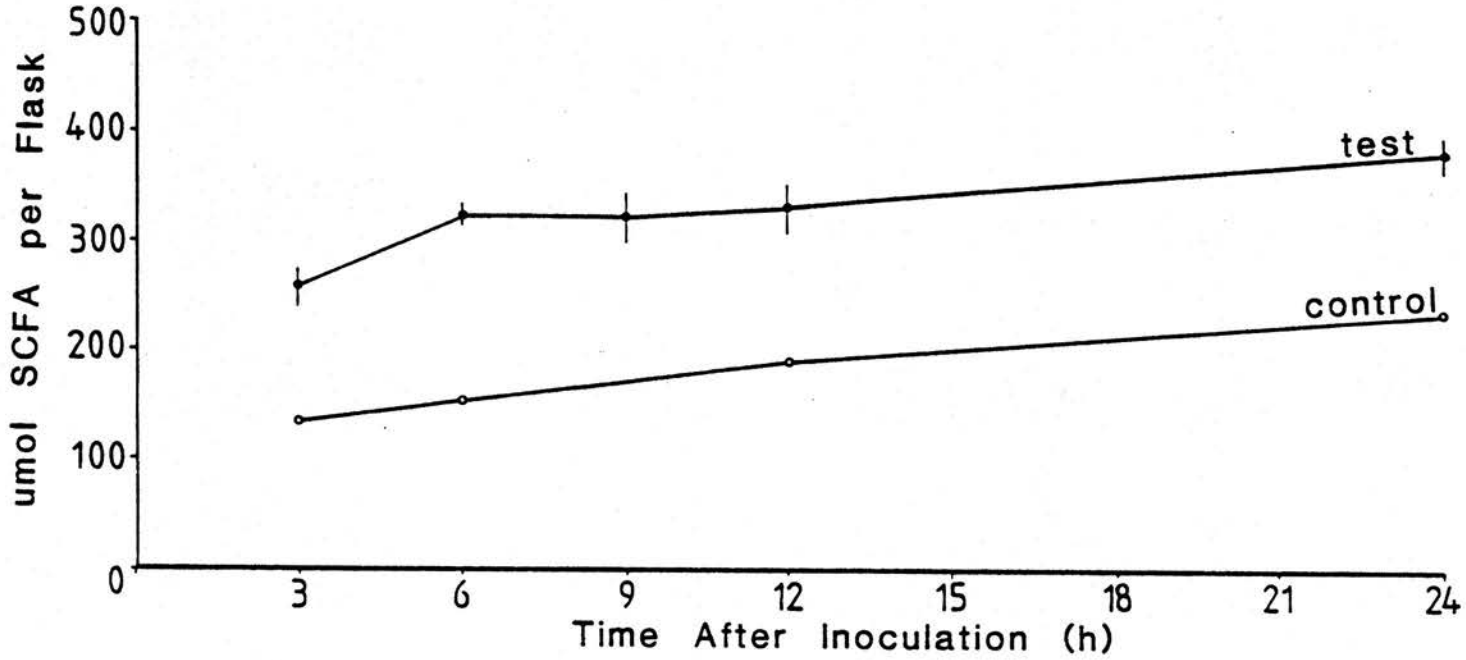

Figure 4.9 : Production of butyrate from *in vitro* fermentations containing ^{14}C-plant cell walls and a high fibre inoculum over 24 hours.

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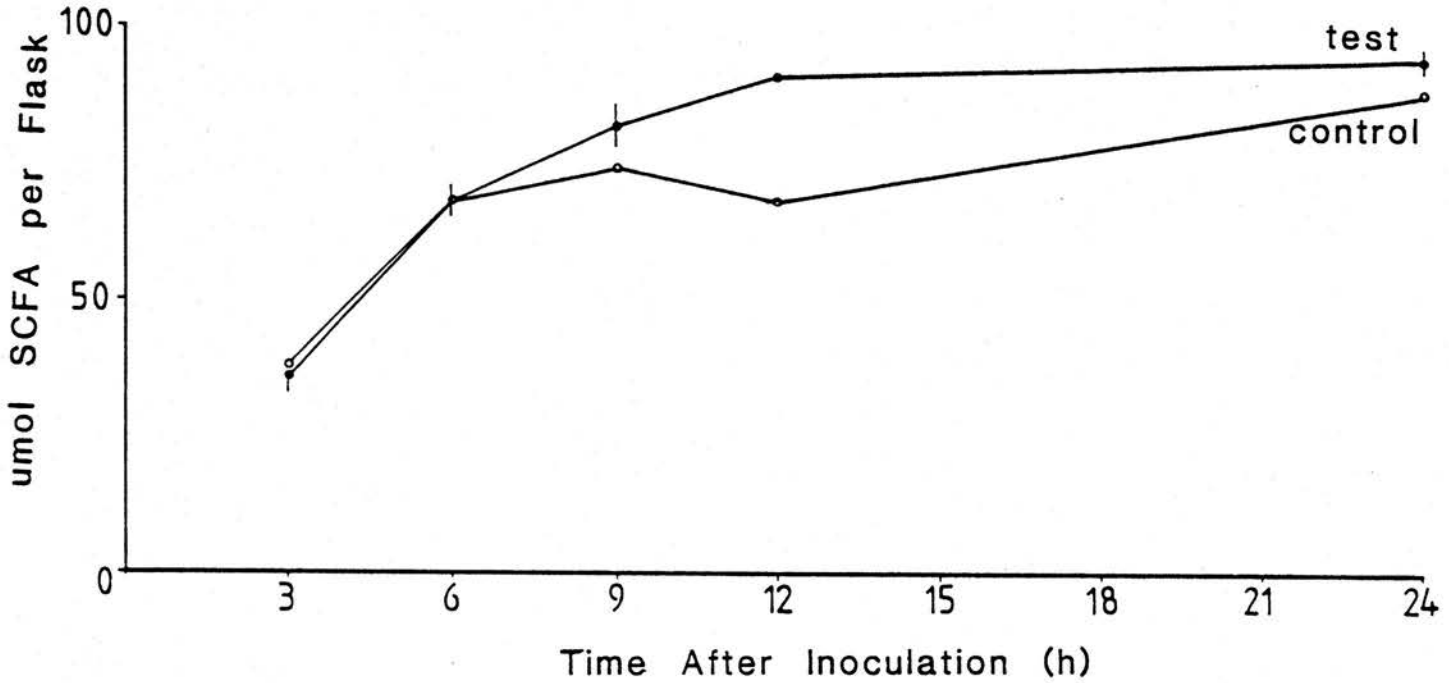

Figure 4.10 : Production of valerate from *in vitro* fermentations containing ^{14}C-plant cell walls and a high fibre inoculum over 24 hours.

Bars = s.e.m.

cell walls to produce SCFA and $^{14}CO_2$ in isolation from the rat. The implications and conclusion of these results in comparison with the complete animal model are discussed in the next chapter.

Fermentation of ^{14}C-Plant Cell Walls in Rats Fed Low Fibre Diets

In order to compare the effect of diet on the fermentation of ^{14}C-plant cell walls, animals were weaned onto and maintained on a low fibre diet (Appendix; Table 2). The results of these studies were based on duplicate or triplicate samples from the same animal and hence extrapolations should be treated with caution. The methods for this investigation are similar to the high fibre investigations although different time intervals were used. The results are given as a percentage of the total ^{14}C dose.

Table 4.3: Distribution of ^{14}C in Rats Fed a Low Fibre Diet

SAMPLES	Time After Gavage (h) 6 (% ^{14}C)	9 (% ^{14}C)	12 (% ^{14}C)	24 (% ^{14}C)
Stomach Contents	5.04	0.58	4.39	0.26
Small Intesttine Contents	24.20	1.35	7.12	0.53
Caecal Contents	23.00	15.62	8.19	5.19
Colon Contents	1.55	30.62	8.67	14.22
Faeces	0.00	0.03	3.54	0.00
TISSUES				
Stomach	0.28	0.16	0.18	0.22
Small Intestines	0.68	0.33	0.64	1.04
Caecum	0.20	0.25	0.17	0.24
Colon	0.11	0.52	0.32	0.71
Liver	1.65	2.16	3.19	3.06
Skin	2.31	0.83	1.97	4.27
Carcass	6.70	9.36	9.95	9.65
$^{14}CO_2$	3.76	3.92	12.21	16.31
TOTAL	69.48	65.73	60.54	55.70

Figure 4.17 shows the production of $^{14}CO_2$ from ^{14}C-plant cell walls in rats maintained on a low fibre diet. From these results, and those in table 4.3, several conclusions were made. Looking at the G.I. contents it was clear that the ^{14}C-plant cell walls were retained in the G.I. tract for a longer period of time than in the animals fed a high fibre diet (Table 4.2). This was also evident from the pattern of $^{14}CO_2$ production (Fig 4.17) in which there was a longer period of constant production of $^{14}CO_2$ between zero and 16 h after gavage, which decreased slightly from 16 to 24 h. However the overall production of $^{14}CO_2$ in the low fibre study (16.3% of the ^{14}C dose) was considerably less than for the high fibre study (26.1% of the ^{14}C dose) after 24 h. These results support the findings of the *in vitro* fermentations, which suggested that the bacterial population in the low fibre fed rats were not adapted to fermentation of dietary polysaccharides. Therefore the bacteria cannot ferment the ^{14}C-plant cell walls to the same extent as the bacterial flora of the high fibre fed rats, despite being in contact with the substrate for longer.

From the remainder of the results in table 4.3 there was incorporation of ^{14}C into various host tissues but unlike the high fibre study there was no subsequent removal. However, the metabolism of ^{14}C from these tissues may occur after the 24 h study period. In these low fibre studies there is need to extend the study period for a longer period. The low numbers of animals used these experiments make the conclusions general and further studies would have to include more animals to assess the effect of a low fibre diet on the activity of the caecal bacteria. It is unlikely that the incorporation of ^{14}C into peripheral tissues (skin and carcass) would show signs of metabolism in an extended trial period as they are long term storage tissues,

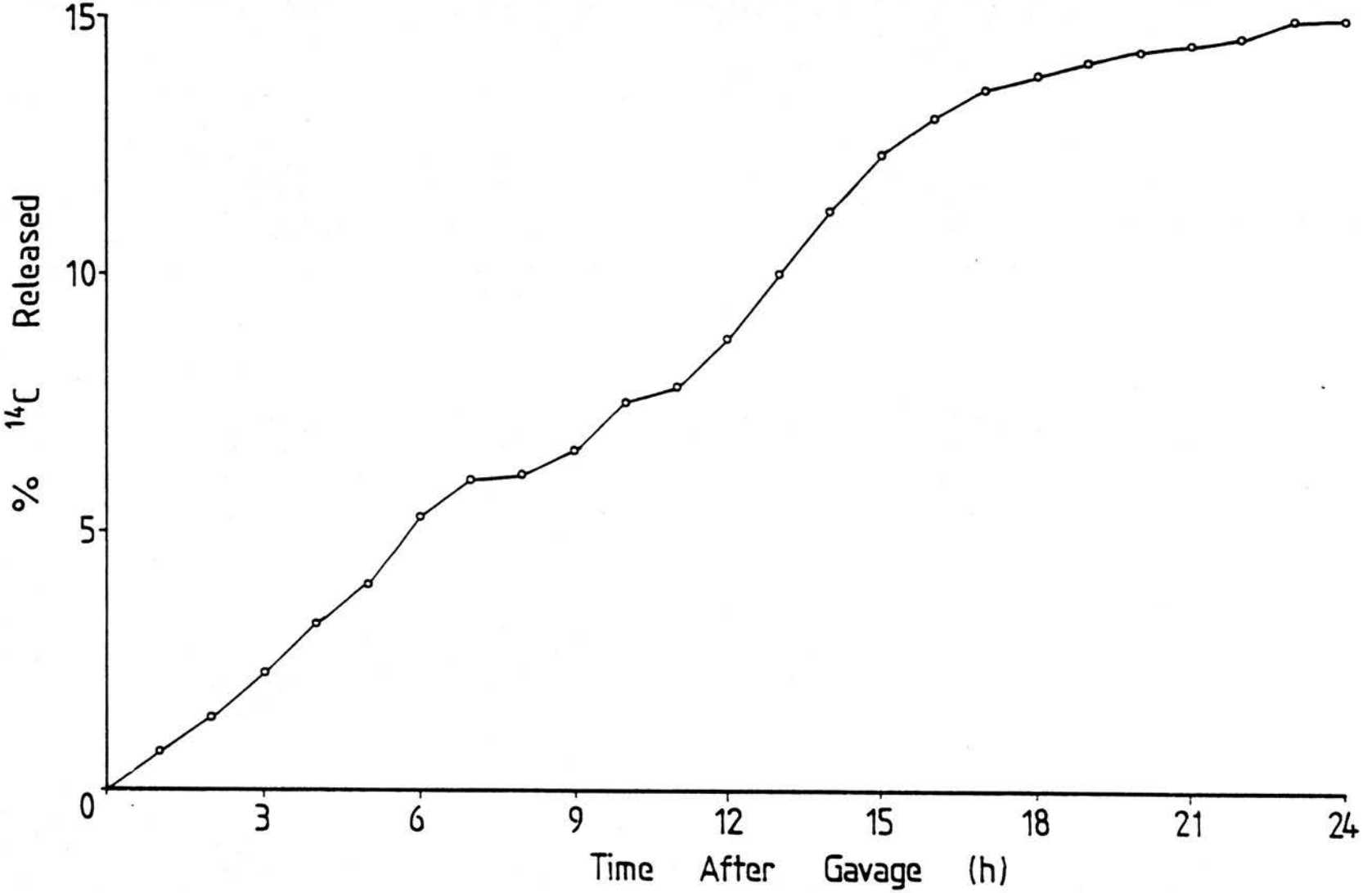

Figure 4.17 : Production of $^{14}CO_2$ from a rat fed a low fibre diet after gavage with ^{14}C-plant cell walls.

although this may occur in other tissue types (especially the colon, caecum and liver), in line with the high fibre study.

Low Fibre Fed Bacterial Fermentations *in Vitro*

The *in vitro* system described was used to investigate the effect of diet on the activity of the caecal bacteria in isolation from the rat. Animals maintained on a low fibre diet were killed at 8 weeks and dissected immediately. Their caeca were isolated and the contents used as a low fibre fed bacterial inoculum for the *in vitro* fermentations.

Figure 4.11 shows the production of $^{14}CO_2$ from this low fibre system and it differs significantly from the $^{14}CO_2$ production graph obtained from the high fibre fermentations (Fig. 4.5). In the high fibre graph the $^{14}CO_2$ production levels off after 12 h and the percentage of the ^{14}C dose released as $^{14}CO_2$ was 14% after 24 h. The low fibre inoculum graph (Fig. 4.11) does not show a reduced $^{14}CO_2$ production between 12 and 24 h and there was still a linear production rate 24 h after inoculation. The amount of ^{14}C released as $^{14}CO_2$ was significantly lower and did not exceed 7% of the initial dose. In the low fibre caecal inoculum there would be a predominance of bacteria which were adapted to utilise the host endogenous secretions (bile, mucus, etc.) as an energy source because very little of the diet would reach the caecum. This would result in a reduced bacterial population with the ability to ferment dietary polysaccharides. Therefore, in the fermentation flasks it would take the DF fermenters longer to become established and consequently fermentation of the ^{14}C-plant cell walls would be slower and drawn out.

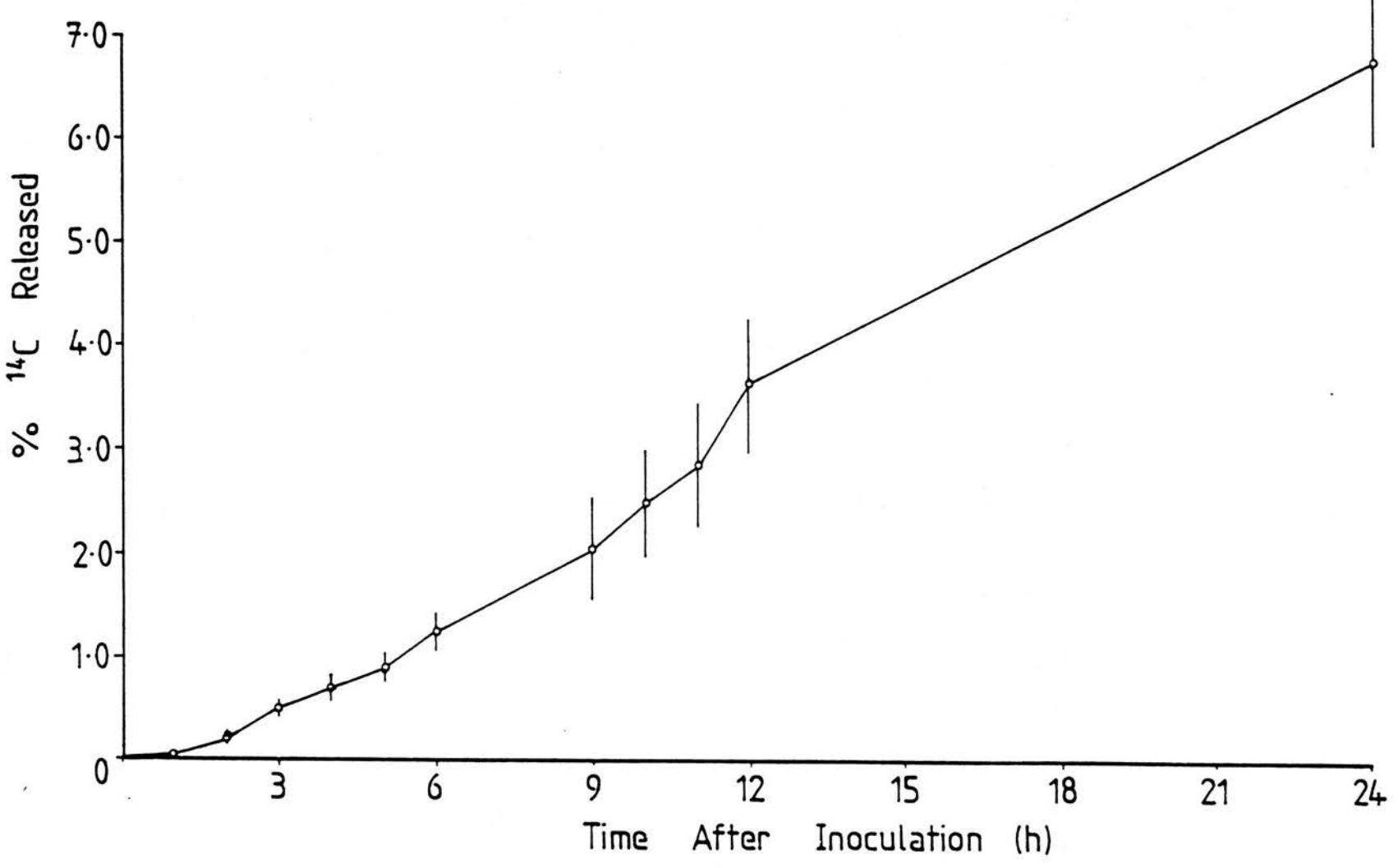

Figure 4.11 : Production of $^{14}CO_2$ from *in vitro* fermentations containing ^{14}C-plant cell walls and a low fibre inoculum over 24 hours. Bars = s.e.m.

The level of the predominant SCFA (acetate) was also indicative of extended fermentation in the culture (Fig. 4.12). There was a continual production of acetate for the entire 24 h which was not seen in the high fibre fermentations, but the control flask (no fibre supplement) showed that acetate production was complete by 12 h. The only other SCFA to show a continual rise in production throughout the 24 h was butyrate (Fig. 4.14). However the increase was not as pronounced as for acetate and was also observed in the control flask (i.e. not arising from fermentation of the ^{14}C-plant cell walls). The most significant difference between the control flask and test flasks was the level of propionate produced. In the test flasks there was almost double the level of propionate than in the control flask (Fig 4.13) and this indicates that propionate was produced from the plant cell walls to a greater extent than the other SCFA. As a consequence, the total SCFA level rises throughout the 24 h period (Fig. 4.16) and was greater in the test flasks compared to the control by approximately a third.

In these low fibre fermentations it was not possible to say whether the breakdown of the ^{14}C-plant cell walls would, if the incubation period was extended, reach the same level as in the high fibre fermentations.

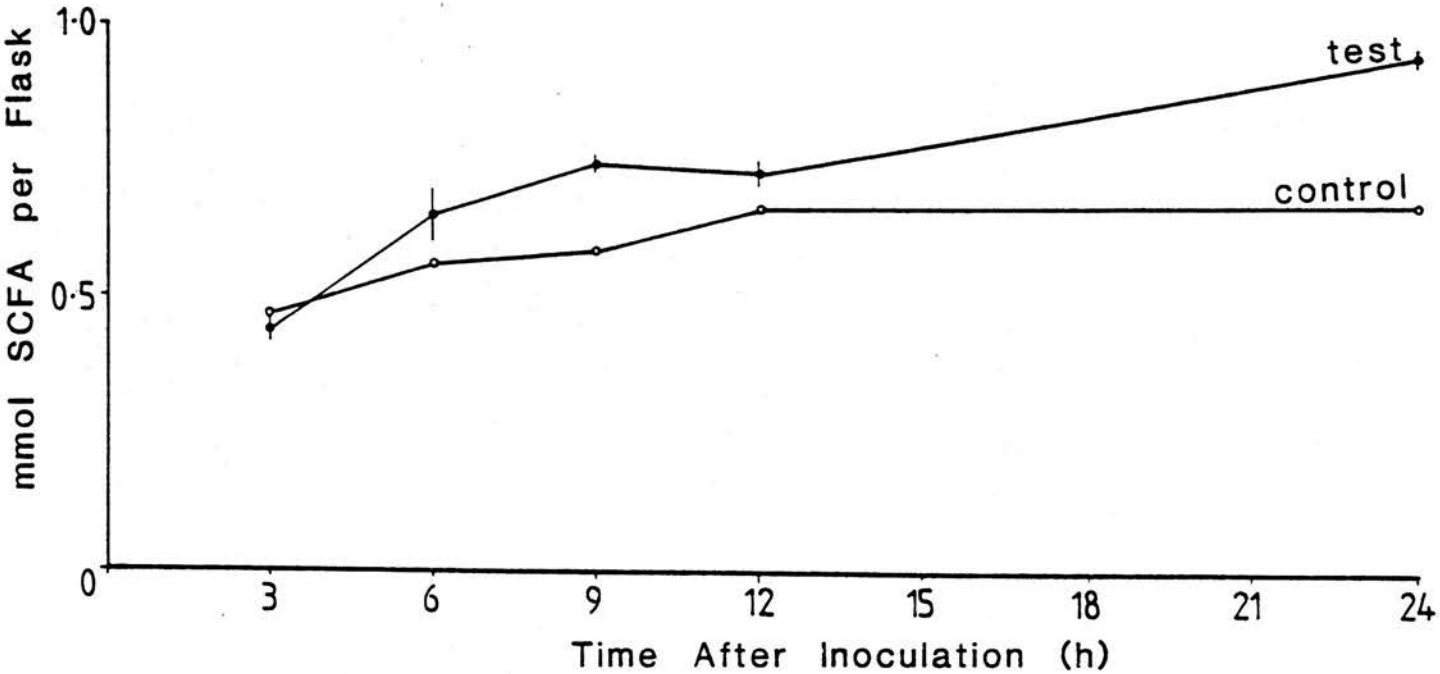

Figure 4.12 : Production of acetate from *in vitro* fermentations containing ^{14}C-plant cell walls and a low fibre inoculum over 24 hours.

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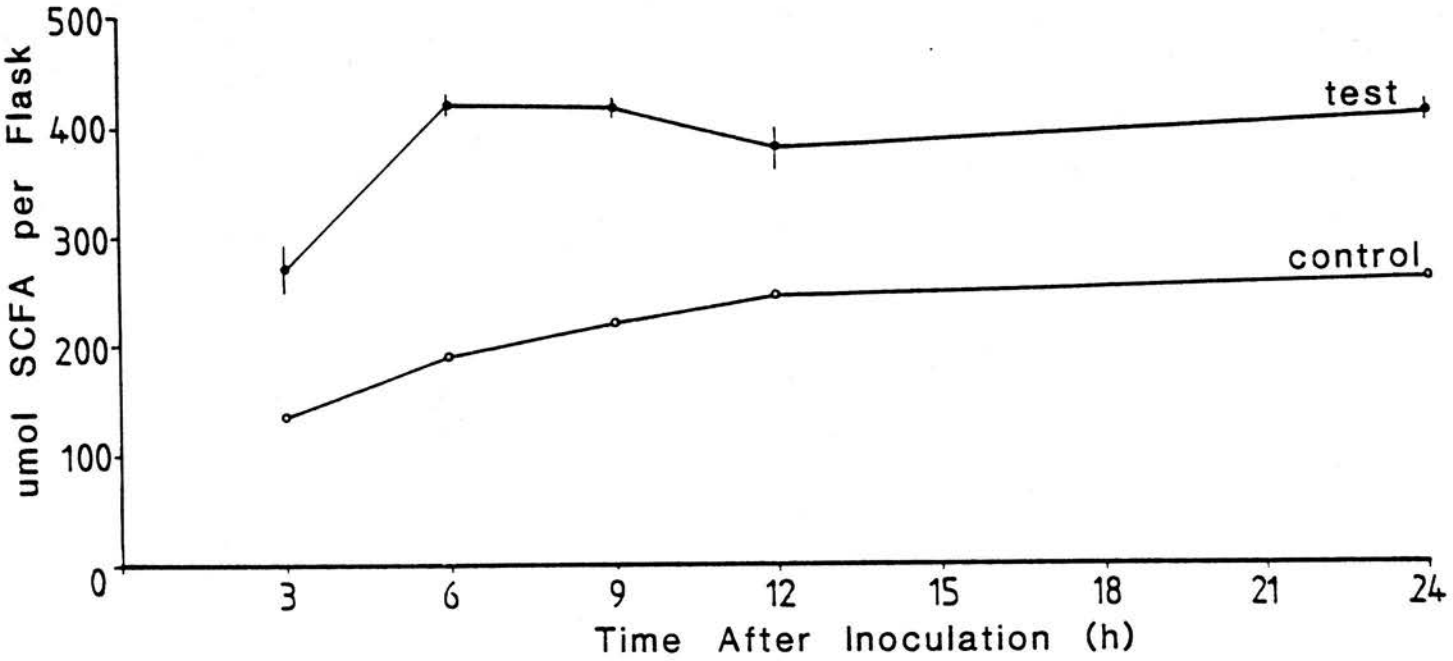

Figure 4.13 : Production of propionate from *in vitro* fermentations containing ^{14}C-plant cell walls and a low fibre inoculum over 24 hours.

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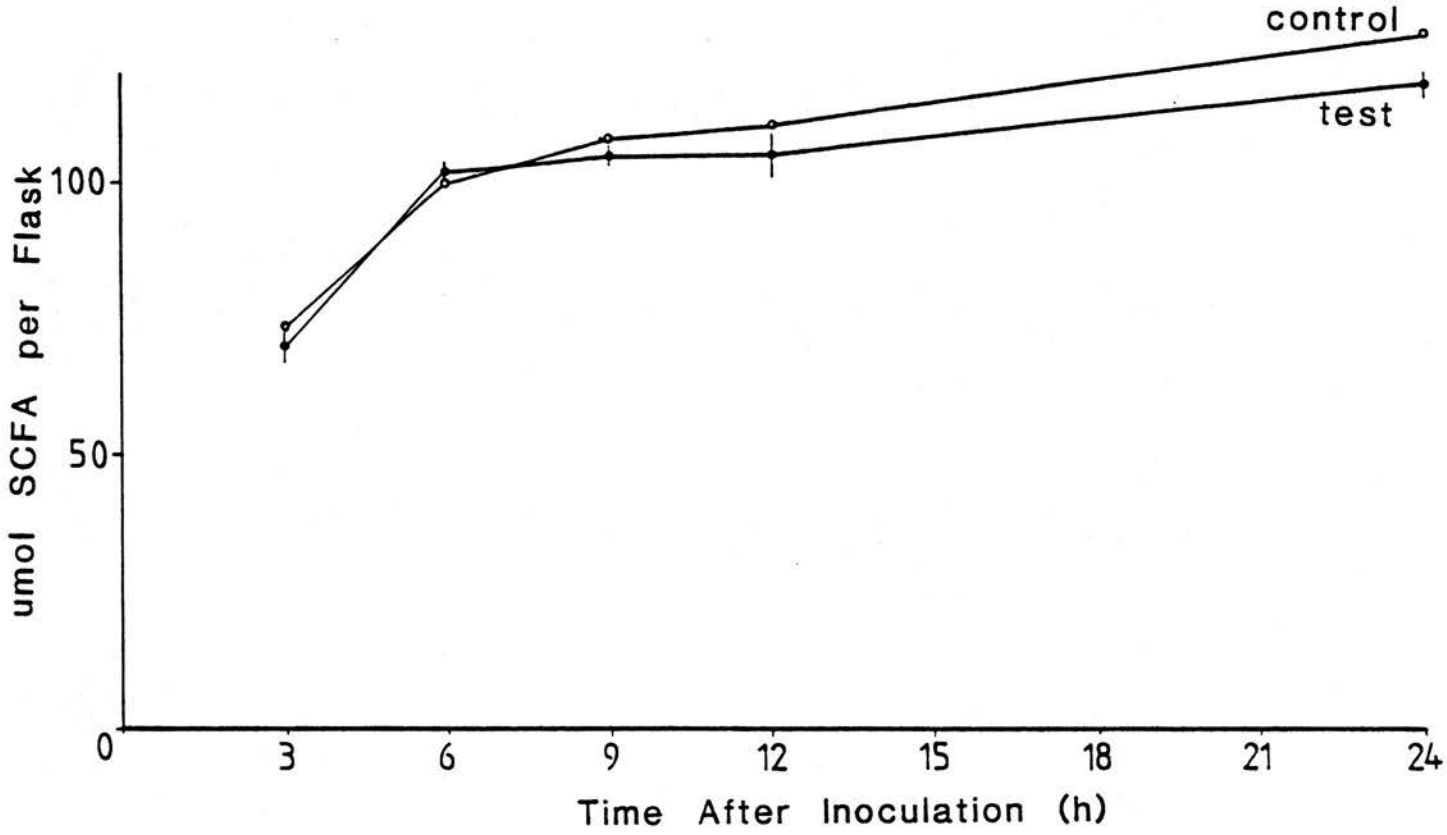

Figure 4.14 : Production of butyrate from *in vitro* fermentations containing ^{14}C-plant cell walls and a low fibre inoculum over 24 hours.

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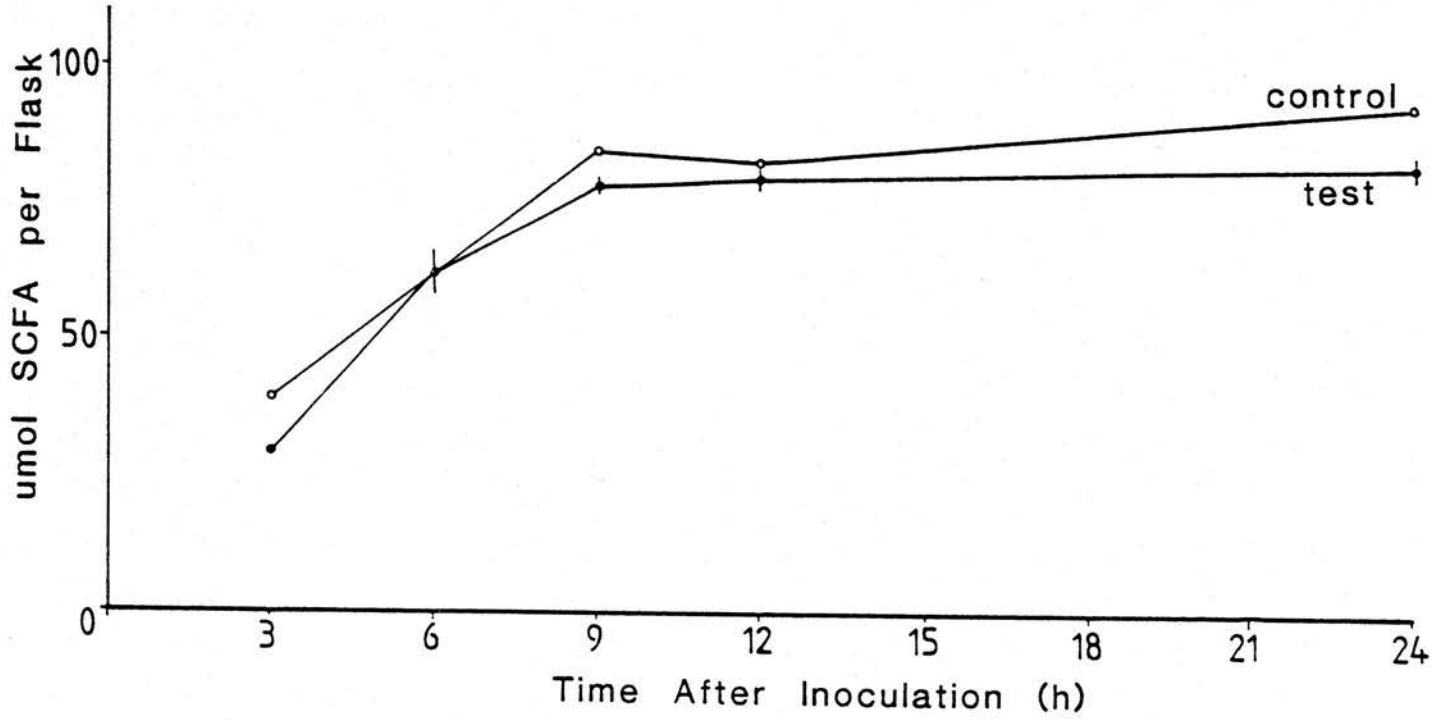

Figure 4.15 : Production of valerate from *in vitro* fermentations containing ^{14}C-plant cell walls and a low fibre inoculum over 24 hours.

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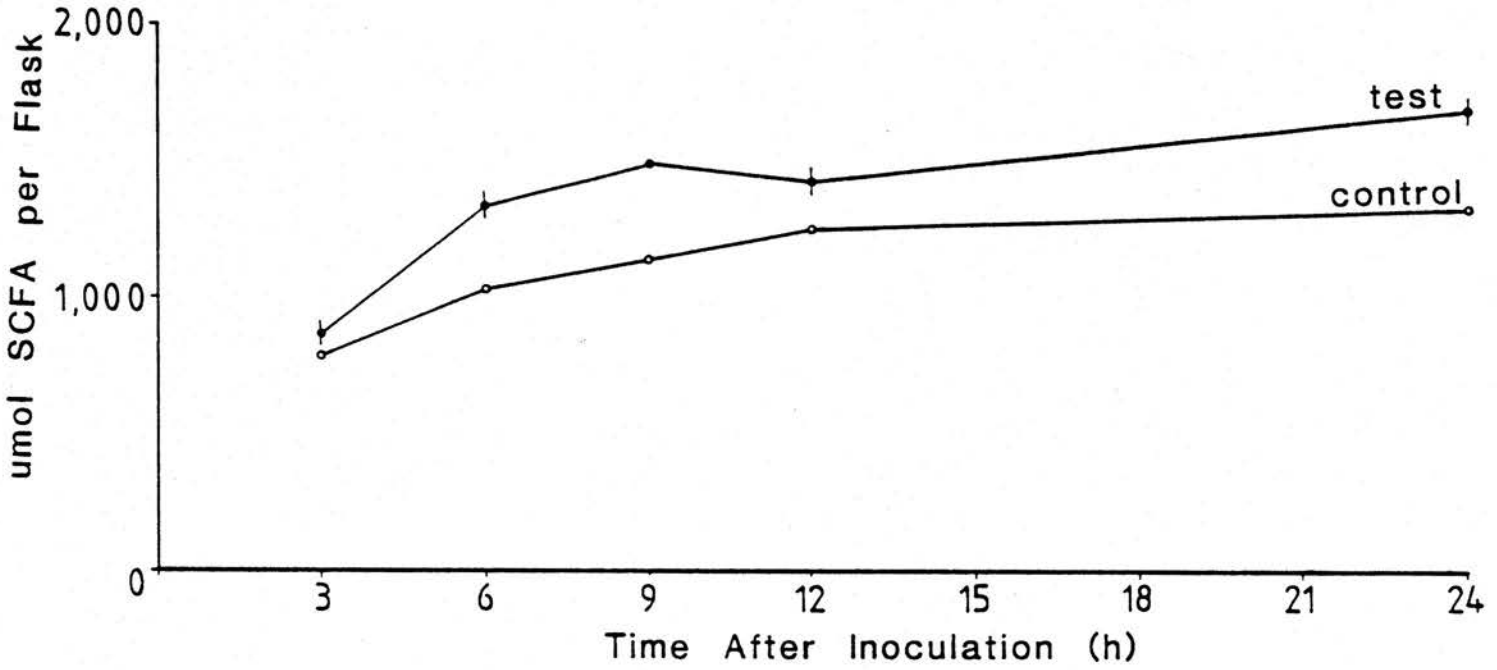

Figure 4.16 : Total production of SCFA from *in vitro* fermentations containing ^{14}C-plant cell walls and a low fibre inoculum over 24 hours.

Bars = s.e.m.

CHAPTER 5 : DISCUSSION

These are the first studies to utilise intact, labelled, plant cell walls as an indicator of the fate of dietary fibre (DF) in the gastrointestinal (G.I.) tract. DF is defined as the polysaccharides and lignin which are indigestible by the host endogenous secretions (Trowell *et al.* 1975) and in our diet the major source is plant cell wall material (Selvendran 1985). However definitions should be treated with caution as they are likely to over simplify the term described. The introductory chapter describes the structure of the cell wall and from that it is difficult to imagine that the polysaccharides and lignin are the only components of the cell wall that are undigested, yet other integral components (e.g. extensin, phenolics, etc.) are degraded in the upper G.I. tract. The enzymes in the stomach and small intestines may not penetrate the cell wall matrix to enable digestion of the integral non-polysaccharide components (extensin) of the cell wall stucture. The Lamport "warp - weft" structure of the cell wall (Lamport and Epstein 1983) would argue against the selective degradation of particular components of the cell wall. Even the more recently introduced term, complex carbohydrate, does not fully explain the component of our diets which reaches the caecum and is available for bacterial fermentation.

There is a fraction of food which is carbohydrate in composition and is undegraded in the upper G.I. tract but its inclusion into the term DF is under debate. "Resistant starch" (RS) is produced during the processing of food and, without prior treatment, is resistant to hydrolysis by amylase (Englyst *et al.* 1987b). The manner of the processing will dictate the level of RS in the food and the same food may have different DF levels depending on the manufacturing procedure. RS should be classified as another source of

fibre in the diet as it becomes available for bacterial fermentation in the caecum/colon. However, caution over its inclusion into the DF term arose from the possible exploitation by food companies. By manipulating food production the level of RS, and hence DF, could be raised to artificially high levels, and may mislead the consumer. These problems arise from the lack of a clear working definition that covers all the components of our diet that reach the caecum and are available for bacterial fermentation.

There are other areas in the field of DF which are hotly disputed and one of these is the methodology for the measurement of DF. This has been reviewed recently by Asp and Johansson (1984). The two main approaches are:

a) Enzymatic/gravimetric methods: The DF content is calculated after removal of the non-fibre components. The Association of Official Analytical Chemists (AOAC) have adopted a variation of this approach in their calculations of the DF content in food.

b) Direct measurement of monomeric DF constituents. The different polysaccharides are extracted and their monomeric composition is analysed by colorimetric or gas-liquid chromatography methods. It is this analysis procedure which is used to determine the DF levels in the British food tables.

The enzymatic/gravimetric method includes RS in its total DF content measurement whereas the direct monomeric evaluation does not. There is a need to determine exactly what should be included under the DF umbrella before deciding which method provides the most accurate measure. The total DF content in a certain food will provide little information about its beneficial effects (bran and pectins have totally different properties in the G.I. tract). Even the monomeric

composition of a specific polysaccharide is a poor predictor of its physiological properties with respect to its interaction with other fibres in the diet. Clearly there is a need for standardisation of many aspects of DF measurement and definition in combination with a knowledge of plant cell wall structure. This may help in predicting how different sources of DF will act in the G. I. tract.

The main aim of this thesis was to investigate, in detail, the fate of undigested plant cell walls in the G. I. tract. Our understanding of the fate of DF in the G. I. tract is limited by the methodology available and these experiments involved the development of a new approach. There are several methodological problems associated with these experiments which have been highlighted throughout this thesis. Despite the faults there are many advantages associated with the use of U-^{14}C-labelled spinach cell walls as a DF marker. The spinach cultures are rapidly growing, undifferentiated cells whose wall structure will be relatively uniform in composition. Cell cultures are easily maintained on a defined medium and by introducing a radioactive precursor in to the medium, labelling of cell wall material is achieved. By using different precursors it is possible to label different polysaccharides within the cell wall matrix or even different residues of the same polysaccharide. The potential for utilisation of these techniques is great and some of the questions to be answered in future work are:

1) What is the fate of isolated polysaccharides in the G. I. tract when compared to chemically identical cell wall bound polysaccharides ?

2) To what extent are cell walls from different plant species degraded in the G. I. tract ? (e. g compare monocots with dicots)

3) Are different sugar residues within the same polysaccharide removed to the same extent ?

In these studies, animals received a mixture of different polysaccharide types (pectins, hemicelluloses and cellulose) when they were fed intact ^{14}C-plant cell walls. This probably reflects more closely the natural state of these polysaccharides as they occur in the normal host diet than in previous studies using isolated fibre sources. The radioactive label is important to reduce the level of substrate required for detection as the ^{14}C-plant cell walls had a high specific activity. This would eliminate any interference with the host diet during the experiment and made possible the comparisons of the fermentation capacity between high and low fibre fed animals. The radioactive marker made the measurement of all DF-derived metabolites possible, irrespective of their destination in the body tissues or expired gases.

In chapter 3, there is a comprehensive analysis of the ^{14}C distribution in the plant cell wall preparation with special interest in the largest fraction , the polysaccharide groups (Table 3.11). The pectic fraction is by far the most abundant and probably consisted of some rhamnogalacturonan (RG) but mostly homogalacturonan. It was assumed that the RG would be RG-I with a repeating rhamnosyl-galacturonic acid backbone. The methyl ester groups in the cell wall are found predominantly on the galacturonic acid residues and the degree of esterification of these residues is important in the three-dimensional structure of the cell wall. The position of the O-acetyl groups has recently been elucidated and they are found on the

galacturonic acid residues of the pectic backbone (Mort *et al.* 1989). From these results the pectin fraction was calculated as approximately 65% homogalacturonan and 35% RG-I. In total, the pectic fraction accounts for nearly half of the total ^{14}C in the plant cell wall preparation.

The hemicellulosic fraction (15.3% of the total ^{14}C) is a relatively minor component when compared to the pectic fraction (48.4% of the total ^{14}C). The two main hemicelluloses are xyloglucan and arabinoxylan and, without the Driselase digestion stage, the two polysaccharides would not be distinguished. All of the xyloglucan-associated xylose would be digested to XG2 by the Driselase, but the arabinoxylan-associated xylose would be converted to the monosaccharide and/or a different disaccharide (xylobiose) which would have a higher R_F value than XG2. Xyloglucan has been shown to contain O-acetyl groups on the nonasaccharide fraction (XG9) but not on the heptasaccharide fraction (XG7) (McNeil *et al.* 1984). The exact location of the O-acetyl groups has recently been resolved and is on the galactose residues (York *et al.* 1988). In arabinoxylan, the O-acetyl groups are attached to the xylose residues of the backbone and all of these substitutions will have important effects in the relative solubility and interaction of the polysaccharides with other cell wall components.

The other fractions quoted in table 3.11 are polysaccharides composed of D-glucose residues. The starch fraction (2.6% of the total ^{14}C) is composed of α-(1→4) glucose residues and the cellulose fraction (21.3% of the total ^{14}C) is composed of β-(1→4) glucose residues. The starch fraction remained even after vigourous treatment with dimethyl sulphoxide (DMSO) and probably became associated with

the cellulose fraction after the cells were disrupted. Starch is poorly digested by Driselase (Fry, personal communication) and will therefore remain in the unknown group of the Driselase soluble fraction (DSF). Cellulose is almost completely digested to monosaccharide by the Driselase treatment and virtually all of the free glucose in the DSF came from cellulose, although some arose from the xyloglucan.

The analysis of the ^{14}C-plant cell wall preparation showed that there was a high pectin content and a high cellulosic content. Selvendran (1985) has expressed concern about the extrapolation from suspension cultures to the primary cell walls of free growing plants. However his critisism is that suspension cultures do not have a middle lamellae and consequently the level of pectin was lower than in the parenchymatous tissues of the plant. Clearly in spinach suspension cultures there is a high pectin content although the level of pectin in the whole plant was not determined. However, the ^{14}C-plant cell walls were not representing the fate of spinach cell walls in the G.I. tract. They were used as a marker for different polysaccharides (homogalacturonan, xyloglucan, cellulose, etc.) to establish the fate of these cell wall bound polysaccharides within the G.I. tract.

The animal studies carried out in this thesis were designed to improve our understanding of the fate of DF in the rat gastro-intestinal tract. The overall view of the project was that the ^{14}C-plant cell walls were broken down extensively by the bacteria of the G.I. tract. Host degradation of the plant cell walls was ruled out by incubating the cell wall preparation with a mixture of digestive enzymes isolated from porcine pancreas. Very little, if any, of the

^{14}C associated with the polysaccharides was released as mono- or di-saccharides after this incubation. If the host enzymic activities were degrading the plant cell walls there would also have been a change in the cell wall polysaccharide composition in the small intestines, a change seen in only the caecum and colon (Fig. 4.3).

From these chromatogaphy profiles it was clear that the bacteria degrade the pectic fraction (galacturonic acid residues) in preference to the cellulose/hemicellulose fraction (glucose residues). Englyst *et al.* (1987a) suggests that there may be a "hierarchy of polysaccharide utilisation" in the large gut which would be primarily a result of the different chemical and physical properties of the individual polysaccharides. If such a heirarchy exists, then it would be reasonable to suggest that the soluble pectins would be more fermentable than the insoluble cellulose by the caecal bacteria.

It is unlikely that the ^{14}C in the faeces after 24 h (17.6% of the total ^{14}C dose) was due entirely to undegraded cellulose as some of the ^{14}C will be incorporated into bacterial components. Hosoya *et al.* (1988) have shown that 10% of the ^{14}C from [^{14}C]fructooligosaccharides incubated with a faecal inoculum will become incorporated into bacterial cells. The extent of fermentation of the fructooligosaccharides was only slightly higher than the ^{14}C-plant cell walls, hence the relative ^{14}C incorporation into bacterial components will be similar. By subtraction, the extent of cellulose degradation in the spinach cell walls was in the range of 40-60%. This level corresponds to values found for the extent of cellulose degradation in the G.I. tract (Cummings 1984).

Bacteria produce carbon dioxide as a product of fermentation and animals produce carbon dioxide as a product of respiration. These two sources of carbon dioxide are present in the animal system described and the only way to evaluate the level of each is to mimic the caecal environment for bacterial fermentations in isolation. *In vitro* fermentations provide important information about bacterial activity in the caecum; however, as Salyers (1984) points out, there should be caution when extrapolating from the *in vitro* system to the *in vivo* animal model. In cetain cases the extrapolation is somewhat premature and requires more investigation. Englyst *et al.* (1987a) suggest that foods with a high starch content will be more beneficial to the host than other dietary polysaccharides because upon fermentation starch produces high butyrate levels. It has been shown that butyrate is the preferential energy source for the colonic mucosa (Roediger 1982) and may be important in the health of the colon with respect to colon cancer (Kruh 1982). However, the debate concerning the level of dietary starch which evades digestion in the upper G.I. tract is still not clear and hence the benefits of foods with a high starch content cannot be inferred from these studies alone. These conclusions also disregard any adverse physiological effects that a high starch diet may exert on the host.

The fermentation system used, in the experiments of this thesis, was based on a minimal salts medium similar to the *in vitro* system of Goering and van Soest (1979). There are many arguments about the choice of medium and the conditions of use e.g. should continuous or batch culture be preferred, should the medium contain endogenous secretions found in the G.I. tract (mucus, bile, etc.), and there is no uniformity on the type of medium used between different research

groups. All of these factors make the comparisons between different experiments difficult but they have to be considered before the choice of system is made.

In these experiments the fermentation capacity of the caecal flora is investigated with respect to the ^{14}C-plant cell walls. The $^{14}CO_2$ produced *in vivo*, from high fibre fed animals, accounts for 26% of the total ^{14}C dose whereas the $^{14}CO_2$ produced with a high fibre inoculum *in vitro* is only 11%. By making a direct comparison between these two experiments there is slightly more $^{14}CO_2$ produced from host metabolism, although the problems associated with such comparisons have been explained. The *in vitro* system may inhibit the bacterial activity by end-product feedback inhibition during the latter stages of the fermentation. However, to my knowledge, this has not been examined in any fermentation systems used to investigate the degradation of polysaccharides.

If a complex medium was used in these experiments (e.g. reinforced clostridium medium) the fermentation of the other components may hide the SCFAs produced from the plant cell walls. In the minimal medium, the SCFA profile is a direct reflection of the fermentation of the plant cell walls. With a high fibre inoculum, production of all the SCFA increases above the control. The bacterial inoculum is primed to a high residue diet in the caecum and hence fermentation capacity is high. The bacteria respond quickly to the *in vitro* situation and the fermention time is relatively short. The two indices of fermentation (SCFA and $^{14}CO_2$ production) level off 12 h after inoculation, suggesting that the limiting factors of the *in vitro* fermentations have been reached.

The control flasks contain only the fibre already in the

inoculum, hence the SCFA profile after 24 h will be similar to that produced in the caecum. The levels of SCFA increase in the test flask above the controls, the relative proportions of SCFA do not alter dramatically. The similar increases of all the SCFAs suggests that the bacterial population remains virtually unaltered in the *in vitro* system throughout the 24 h trial period. However, different bacterial populations could also produce similar SCFA profiles but would take time to become established.

The comparison of a high and low fibre fed inoculum for *in vitro* fermentations emphasises the differences between the two bacterial populations. The low fibre inoculum does not have the same capacity to ferment the ^{14}C-plant cell walls and as a result less $^{14}CO_2$ is produced. The level of SCFA produced is also considerably lower in the low fibre inoculum (Fig. 4.16) than in the high fibre inoculum (Fig. 4.6). To examine the reason behind these effects, an in depth microbiological study would be required to determine the dominant species and relative numbers in the respective caecal populations.

Wolin and Miller (1983) describe how the different bacterial species interact to degrade cellulose in the rumen. It is virtually certain that these interactions occur in other anaerobic environments (e.g the caecum/colon) to degrade different polysacharides. In the low fibre fed inoculum, the bacteria have had virtually no exposure to fibre and the population will have been adapted to utilisation of the host secretions as energy sources. The bacterial population will be geared towards the break down of substrates other than plant cell wall material and, as a consequence, the fermentation capacity of this inoculum is greatly reduced. The acetate and propionate levels in the

test flask of the low fibre inoculum are higher than the control flask suggesting that there are bacteria present which will ferment the ^{14}C-plant cell walls to these SCFA in preference to the other SCFA. It is unlikely that one bacterial species could produce SCFA from the fermentation of plant cell wall material without the assistance of other bacterial species. It is more likely that there are distinct bacterial populations within the caecum which will adapt quickly to the change in environment and utilise the available substrates.

After production in the caecum, SCFA are readily absorbed through the colonic epithelium and it has been suggested that they contribute significantly to the energy intake of the host (Høverstad *et al.* 1982). In the low fibre inoculum there is production of propionate and this is thought to be important in gluconeogenesis and cholesterolneogenesis in the liver (Chen *et al.* 1984). The other SCFA produced, acetate, is not removed by the liver and is probably circulated to peripheral tissues where it is metabolised (Knowles *et al.* 1974). It is probably by these mechanisms that the ^{14}C becomes incorporated into different host tissues. However, to draw far reaching conclusions about the possible beneficial effects a polysaccharide may confer on the host from *in vitro* studies requires caution.

The small number of studies on the fate of ^{14}C-plant cell walls in rats fed a low fibre diet completed the comparisons of diet and incubation techniques (i.e. *in vivo*/*in vitro* and high/low fibre). The $^{14}CO_2$ produced from low fibre fed animals (Fig. 4.17) follows a similar pattern to the high fibre fed animals (Fig. 4.4) except that the period of greatest production is extended. This is also found *in*

vitro from the low fibre inoculum where it is thought that the bacterial enzymes for polysaccharide break down were induced. The low fibre fed animals will have a slower whole gut transit, hence the ^{14}C-plant cell walls will remain in the caecum and colon for longer. Even with this time advantage the bacteria cannot ferment the plant cell wall material as efficiently as the high fibre fed bacteria presumably because the numbers and species required are not dominant in the caeca of these animals and take time to adapt.

The capacity of animals to produce $^{14}CO_2$ from ^{14}C-plant cell walls when maintained on a high or low fibre diet was examined in chapter 4. Although the relationship between the production of $^{14}CO_2$ *in vitro* and *in vivo* has not been completely resolved, certain assumptions and conclusions can be made. Production of $^{14}CO_2$ by the host could only result from the absorption and respiration of ^{14}C-SCFA after fermentation by the bacterial flora. Although animals maintained on high fibre diets produce more than double the level of $^{14}CO_2$ than animals maintained on low fibre diets, the ratio of *in vitro* to *in vivo* $^{14}CO_2$ production remains the same. This would suggest that the capacity of the host to absorb and utilise the bacterial fermentation products (^{14}C-SCFA) is unaffected by the diet. A similar proportion of SCFA are absorbed and metabolised in both high and low fibre fed animals irrespective of the amount produced although the actual proportion of SCFA utilised is not known. The point should be stressed that these are results from animals that were 6-8 weeks old and their ability to absorb and utilise SCFAs from the colon may alter with age.

REFERENCES

Anderson J.W. and Bridges S.R. (1984) Short-chain fatty acid fermentation products of plant fiber affect ,glucose metabolism of isolated rat hepatocytes.
Proc. Soc. Exp. Biol. Med. **177**: 372-376

Asp N-G. and Johansson C-G. (1984) Dietary fibre analysis.
Nutr. Abstr. Rev. Clin. Nutr. **54**: 735-751

Bond J.H. and Levitt M.D. (1976) Fate of soluble carbohydrate in the colon of rats and man.
J. Clin. Invest. **57**: 1158-1164

Bonhomme-Florentin A. (1988) Degradation of hemicellulose and pectin by horse caecum contents.
Br. J. Nutr. **60**: 185-192

Burkitt D.P. (1972) Varicose veins, deep vein thrombosis, and haemorrhoids: Epidemiology and suggested aetiology.
Br. Med. J. 2: 556-561

Burkitt D.P. (1973) Epidemiology of large bowel disease: the role of fibre.
Proc. Nutr. Soc. **32**: 145-149

Carryer P.W., Brown M.L., Malagelada J-R., Carlson G.L. and McCall J.T Quantification of the fate of dietary fiber in humans by a newly developed radiolabeled fiber marker.
Gastroenterology **82**: 1389-1394

Chen W-G. L., Anderson J.W. and Jennings D. (1984) Propionate may mediate the hypocholesterolaemic effects of certain soluble plant fibres in cholesterol-fed rats.
Proc. Soc. Exp. Biol. Med. **175**: 215-218

Cleave T.L. (1974) The Saccharine Disease. Wright, Bristol.

Cooper J.B. (1984) Hydroxyproline synthesis is required for cell wall recognition.
In Structure, Function and Biosynthesis of Plant Cell Walls (eds. W.M. Dugger & S. Bartniki-Garcia) Am. Soc. Plant Physiol.

Cummings J.H. (1983) Fermentations in the human large intestines: Evidence and implications for health.
Lancet 1: 1206-1209

Cummings J.H. (1984) Cellulose and the human gut.
Gut **25**: 805-810

Cummings J.H. and Branch W.J. (1984) Fermentation and the production of short-chain fatty acids in the human large intestines.
In: Dietary Fiber, Basic and Clinical Aspects. (Eds. Vahouny G.V. and Kritchevsky D.) Plenum Press, New York.

Cummings J.H. and Englyst H.N. (1987) Fermentation in the human large intetine and the available substrates.
Am. J. Clin. Nutr. **45**: 1243-1255

Eastwood M.A. (1987) Dietary fiber and the risk of cancer.
Nutr. Rev. **45**: 193-198

Eastwood M.A. and Brydon W.G. (1985) Physiological effects of dietary fibre on the alimentary tract.
In Dietary Fibre, Fibre-Depleted Foods and Disease. Academic Press Inc. London.

Eastwood M.A., Elton R.A. and Smith J.H. (1986) Long-term effect of wholemeal bread on stool weight, transit time, fecal bile acids, fats, and neutral sterols.
Am. J. Clin. Nutr. **43**: 343-349

Edwards C.A., Blackburn N.A., Craigen L., Davison P., Tomlin J., Sugden K., Johnson I.T. and Read N.W. (1987) Viscosity of food gums determined in vitro related to their hypoglycemic actions.
Am. J. Clin. Nutr. **46**: 72-77

Englyst H.N., Hay S. and Macfarlane G.T. (1987a) Polysaccharide breakdown by mixed populations of human faecal bacteria.
FEMS Microbiol. Ecol. **95**: 163-171

Englyst H.N., Trowell H., Southgate D.A.T. and Cummings J.H. (1987b) Dietary fibre and resistant starch.
Am. J. Clin. Nutr. **46**: 873-874

Fry S.C. (1982a) Phenolic components of the primary cell wall.
Biochem. J. **203**: 493-504

Fry S.C. (1982b) Isodityrosine, a new cross-linking amino acid from plant cell wall glycoprotein.
Biochem. J. **204**: 449-55

Fry S.C. (1985) Primary cell wall metabolism.
Oxford Surv. Plant Molec. Cell Biol. **2**: 1-42

Fry S.C. (1988) The growing plant cell wall: Chemical and metabolic analysis. Longman, London.

Goering H.K. and Van Soest P.J. (1970) Forage Fibre Analyses (Apparatus, Reagents, Procedures and Some Applications).
Agriculture Handbook 379, U.S. Department of Agriculture

Grimble G.K. Patil D.H. and Silk D.B.A. (1988) Assimilation of lactitol, an 'unabsorbed' disaccharide in the normal human colon.
Gut **29**: 1666-1671

Green J.R. and Northcote D.H. (1979) Location of a fucosyltransferase in the membrane system of maize root cells.
J. Cell Sci. **40**: 235-44

Hidaka H., Eida T., Takizawa T., Tokunaga T. and Tashiro Y. (1986) Effects of fructooligosaccharides on intestinal flora and human health.
Bifidobacteria Microflora **5**: 37-50

Hosoya N., Dhorranintra B. and Hidaka H. (1988) Utilization of [U-^{14}C]fructooligosaccharides in man as energy resources.
J. Clin. Biochem. Nutr. **5**: 67-74

Høverstad T., Bøhmer T. and Fausa O. (1982) Absorption of short-chain fatty acids from the human colon measured by the $^{14}CO_2$ breath test.
Scand. J. Gastroenterol. **17**: 373-378

Judd P.A. and Truswell A.S. (1985) The hypocholesterolaemic effects of pectin in rats.
Br. J. Nutr. **53**: 409-425

Kelleher J., Walters M.P., Srinivasan G.R., Hart G., Findlay J.M. and Losowsky M.S. (1984) Degradation of cellulose within the gastrointestinal tract in man.
Gut **25**: 811-815

Knowles S.E., Jarret I.G., Filsell O.H. and Ballard F.J. (1974) Production and utilisation of acetate in mammals.
Biochem. J. **142**: 401-411

Kruh J. (1982) Effects of sodium butyrate, a new pharmacological agent, on cells in culture.
Molec. Cellular Biochem. **42**: 65-82

Lamport D.T.A and Epstein L. (1983) A new model for the primary cell wall: A concatenated extensin-cellulose network.
Current Topics Plant Biochem. Physiol. **2**: 73-82

McBurney M.I., Horvath P.J., Jeraci J.L. and Van Soest P.J. (1985) Effect of an *in vitro* fermentation using human faecal inoculum on the water holding capacity of dietary fibre.
Br. J. Nutr. **53**: 17-24

McBurney M.I. and Thompson L.U. (1987) Effect of human faecal inoculum on *in vitro* fermentation variables.
Br. J. Nutr. **58**: 233-243

McDougall G.J. and Fry S.C. (1988) Inhibition of auxin-stimulated growth of pea stem segments by a specific nonasaccharide of xyloglucan.
Planta **175**: 412-416

McLean Ross A.H., Eastwood M.A., Brydon W.G., Anderson J.R. and Anderson D.M.W. (1983) A study of the effects of dietary gum arabic in humans.
Am. J. Clin. Nutr. **37**: 368-375

McNeil M., Albersheim P., Taiz L. and Jones R.L. (1975) The structure of plant cell walls VII. Barley aleurone cells.
Plant Physiol. **55**: 64-68

McNeil M., Darvill A.G., Fry S.C. and Albersheim P. (1984) Structure and function of the primary cell walls of plants.
Ann. Rev. Biochem. **53**: 625-63

Markwalder H-U. and Neukom H. (1976) Diferulic acid as a possible crosslink in hemicelluloses from wheat germ.
Phytochem. **15**: 836-837

Melton L.D., McNeil M., Darvill A.G., Albersheim P. and Dell A. (1986) Structural characterization of oligosaccharides isolated from the pectic polysaccharide rhamnogalacturonan II.
Carbohy. Res. **146**: 279-305

Miller T.L. and Wolin M.J. (1979) Fermentations by saccharolytic intestinal bacteria. Am. J. Clin. Nutr. **32**: 164-172

Mort A.J., Komalavilas P., Rorrer G.L. and Lamport D.T.A. (1989) Anhydrous hydrogen fluoride and cell wall analysis.
In: Plant Fibers (Eds. H.F. Linskens and J.F. Jackson) Springer-Verlag, Berlin.

Nyman M., Asp N-G, Cummings J. and Wiggins H. (1986) Fermentation of dietary fibre in the intestinal tract: Comparison between man and rat.
Br. J. Nutr. **55**: 487-496

Painter N.S. (1975) Diverticular Disease of the Colon: A Deficiency Disease of Western Civilisations. Heineman, London.

Preston R.D. (1974) The Physical Biology of Plant Cell Walls. London. Chapman & Hall.

Prynne C.J. and Southgate D.A.T. (1979) The effects of a supplement of dietary fibre on faecal excretion in human subjects.
Br. J. Nutr. **41**: 495-503

Rees D.A. and Wright A.W. (1971) Polysaccharide conformation part VII. Model building computations for ($\alpha 1 \rightarrow 4$) galacturonan and the kinking function of L-rhamnose residues in pectic substances.
J. Chem. Soc. (B) 1366

Roediger W.E.W. (1982) Utilization of nutrients by isolated epithelial cells of the rat colon.
Gastroenterol. **83**: 424-429

Roediger W.E.W. and Moore A. (1981) Effect of short-chain fatty acids in sodium absorption in isolated human colon perfused through the vascular bed.
Dig. Dis. Sci. **26**: 100-106

Ruppin H., Bar-Meir S., Soergel K.H., Wood C.M. and Schmitt M.G. (1980) Absorption of short-chain fatty acids by the colon.
Gastroenterology **78**: 1500-1507

Sakata T. (1987) Stimulatory effect of short-chain fatty acids on epithelial cell proliferation in the rat intestine: A possible explanation for trophic effects of fermentable fibre, gut microbes and luminal trophic factors.
Br. J. Nutr. **58**: 95-103

Salyers A.A. (1984) Diet and the colonic environment.
In Dietary Fiber: Basic and Clinical Aspects. (Eds. Vahouny G.V. and Kritchevsky D.). Plenum Press. New York.

Selvendran R.R. (1984) The plant cell wall as a source of dietary fibre: Chemistry and structure.
Am. J. Clin. Nutr. **39**: 320-337

Selvendran R.R. (1985) Developments in the chemistry and biochemistry of pectic and hemicellulosic polymers.
J. Cell Sci. Suppl. **2**: 51-88

Selvendran R.R. (1987) Chemistry of plant cell walls and dietary fibre.
Scand. J. Gastroent. **22**: Suppl. 129 33-41

Singleton P. and Sainsbury D. (1978) Dictionary of Microbiology. Wiley and Sons Ltd., Chichester.

Spillar G.A., Chernoff M.C., Hill R.A., Gates J.E., Nassar J.J. and Shipley E.A. (1980) Effect of purified cellulose, pectin and a low residue diet on fecal volatile fatty acids, transit time and fecal weight in humans.
Am. J. Clin. Nutr. **33**: 754-759

Stephen A.M. and Cummings J.H. (1979) Water-holding by dietary fibre *in vitro* and its relationship to faecal output in man.
Gut **20**: 722-729

Stephen A.M., Wiggins H.S. and Cummings J.H. (1987) Effect of changing transit time on colonic microbial metabolism in man.
Gut **28**: 601-609

Tomlin J. and Read N.W. (1988) Laxative properties of indigestible plastic particles.
Br. Med. J. **297**: 1175-1176

Tomlin J., Read N.W., Edwards C.A. and Duerden B.I. (1986) The degradation of guar gum by a faecal incubation system.
Br. J. Nutr. **55**: 481-486

Trowell H.C. (1975) Dietary-fiber hypothesis of the etiology of diabetes mellitus.
Diabetes **24**: 762-765

Trowell H.C. and Burkitt D.P. (1987) The development of the concept of dietary fibre.
In Dietary Fibre in Health and Disease. (Eds. McLean Baird I. and Ornstein M.H.). Pergamon Press, Oxford.

Trowell H.C., Southgate D.A.T., Wolever T.M.S., Leeds A.R., Gassul M.A. and Jenkins D.J.A. (1976) Dietary fibre redefined.
Lancet **1**: 967 (letter)

Van Soest P.J., Jeraci J., Foose T., Wrick K. and Ehle F. (1983) Comparative fermentation of fibre in man and other animals.
In: Proceedings of Fibre in Human and Animal Nutrition Symposium, pp 75-80 (Eds. Wallace G. and Bell L.) **Bulletin 20**. The Royal Society of New Zealand, Wellington, New Zealand.

Von Engelhardt W. and Rechkemmer G. (1983) The physiological effects of short-chain fatty acids in the hind gut.
In: Proceedings of Fibre in Human Health and Animal Nutrition Symposium, pp 149 (Eds. Wallace G. and Bell L.) **Bulletin 20.** The Royal Society of New Zealand, Wellington, New Zealand.

Walters M.P., Kelleher J., Findlay J.M. and Srinivasan S.T. (1989) Preparation and characterization of a [^{14}C]cellulose suitable for human metabolic studies.
Br. J. Nutr. **62**: 121-129

Wilson L.G. and Fry J.C. (1986) Extensin: a major cell wall glycoprotein Plant Cell Environ. **9**: 239-260

Wolin M.J. (1974) Metabolic interactions among intestinal microorganisms.
Am. J. Clin. Nutr. **27**: 1320-1328

Wolin M.J. and Miller T.L. (1983) Interactions of microbial populations in cellulose fermentation.
Federation Proc. **42**: 109-113

Yajima T. (1985) Contractile effects of short-chain fatty acids on the isolated colon of the rat.
J. Physiol. **368**: 667-678

York W.S., Oates J.E., van Halbeek H., Darvil A.G., Albersheim P., Tiller P.R., Dell A. (1988) Location of the O-acetyl substituents on a nonasaccharide repeating unit of sycamore extracellular xyloglucan.
Carb. Res. **173**: 113-132.

APPENDIX

TABLE 1: Plant Cell Culture Medium

Compound	Concentration (mg/l)
Glucose	10,000
KNO_3	1900
NH_4NO_3	1650
$CaCl_2 . 2H_2O$	437
$MgSO_4 . 7H_2O$	370
KH_2PO_4	170
$MnSO_4 . 4H_2O$	22.5
$ZnSO_4 . 7H_2O$	10.4
$Na_2EDTA . 2H_2O$	7.4
H_3BO_3	6.2
$FeCl_3 . 6H_2O$	5.4
KI	0.83
$NaMoO_4 . 2H_2O$	0.25
$CoCl_2 . 6H_2O$	0.025
$CuSO_4 . 5H_2O$	0.025

pH 4.4 with 1 M HCl

Adapted from the medium used by Fry (1982a)

TABLE 2: Detailed Analysis of CRM(X) Diet

Proximate Analysis	
Crude Oil	2.9%
Crude Protein	18.3%
Crude Fibre	3.5%
Calcium (as Ca)	0.8%
Phosphorous (as P)	0.6%
Salt	0.7%
Carbohydrate	56.3%
Metabolisable Energy	2916kcal/kg

Trace Elements Added	
Cobalt	0.4 ppm
Copper	7 ppm
Iodine	1.3 ppm
Iron	30 ppm
Magnesium	102 ppm
Manganese	25 ppm

Amino Acids (% of diet)	
Arginine	1.2%
Cysteine	0.2%
Glycine	0.9%
Histidine	0.4%
Isoleucine	0.7%
Leucine	1.4%
Lysine	1.0%
Methionine	0.3%
Phenylalanine	0.8%
Threonine	0.6%
Tryptophan	0.2%
Tyrosine	0.6%
Valine	0.8%

Vitamins Added per kg	
Vitamin A	8,000 iu
Vitamin B_1	4 mg
Vitamin B_2	8 mg
Vitamin B_6	6 mg
Vitamin B_{12}	12 µg
Vitamin D_3	1,000 iu
Vitamin E	60 iu
Vitamin K	10 mg
Choline chloride	200 mg
Folic acid	10 mg
Pantothenic acid	12 mg

Information supplied by Special Diet Services Ltd.

TABLE 3: Comparison Between High and Low Fibre Diets

Low Fibre Diet		High Fibre Diet - CRM(X)	
Component	**% Feed**	**Component**	**% Feed**
Commercial pregelatinised wheat starch	71.2	Crude fibre (Wheat fibre)	3.6
Wheat gluten	8.9	Crude protein	18.1
Caesin	8.0	Carbohydrate	56.9
Dried egg	4.0	Crude oil	2.4
L-lysine hydrochloride	0.3	L-leucine	1.4
L-threonine	0.1	L-lysine	1.0
Corn oil	2.0	Calcium	0.8
Vitamin mix	1.0	Vitamin mix	1.0
Mineral mix	4.5	Mineral mix	4.5
Supplied in a powdered form and then mixed to a paste		Supplied in pelleted form	

All information supplied by Special Diet Services Ltd.

TABLE 4: Fermentation Medium

Buffer solution:	
Distilled water	18 l
Ammonium bicarbonate	72 g
Sodium bicarbonate	630 g

Macromineral solution:	
Distilled water	1 l
Na_2HPO_4, anhydrous	5.7 g
KH_2PO_4, anhydrous	6.2 g
$MgSO_4.7H_2O$	0.6 g

Micromineral solution:	
$CaCl_2.2H_2O$	13.2 g
$MnCl_2.4H_2O$	10.0 g
$CoCl_2.6H_2O$	1.0 g
$FeCl_3.6H_2O$	8.0 g

Distilled water up to 100 ml

Reducing solution:	
Distilled water	95 ml
Cysteine hydrochloride	625 mg
1 M NaOH	4 ml
Sodium sulphide. $9H_2O$	625 mg

To prepare the medium add, in order, 2 g of tryptone, 400 ml of distilled water and 0.1 ml of micromineral solution, and agitate to dissolve. Then add 200 ml of buffer solution, 200 ml of macromineral solution and 1 ml of a 0.1% (w/v) resazurin solution. Mix and add 40 ml to each 100 ml flask.

Adapted from Goering and Van Soest (1979).